

DECIPHERING THE ROLE OF DNMT3B IN REGULATING DNA METHYLATION
AND EPIGENETIC MODULATION OF TRANSCRIPTIONAL SILENCING

By

Khadijah A. Mitchell

A dissertation submitted to Johns Hopkins University in conformity with
the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
July 2014

© Khadijah A. Mitchell 2014
All Rights Reserved

Abstract

DNA methylation is one of several epigenetic mechanisms used by cells to control gene expression. DNA methylation patterns are not randomly distributed, instead they are compartmentalized by region. In normal cells heterochromatin is hypermethylated and transcriptionally silent. The rest of the genome is predominantly CpG poor, except for CpG islands, which tend to be hypomethylated and associated with active genes. In tumors this normal compartmentalization is reversed. Cancers are characterized by global hypomethylation that affects repetitive DNA found in heterochromatin, leading to genomic instability. Concurrently, site-specific hypermethylation of CpG islands at the promoters of tumor suppressor genes leads to silencing.

This work focused on how DNMT3B influences methylation patterns on a global and local scale. We hypothesized DNMT3B regulated DNA methylation outside of promoter regions. Using an isogenic colorectal cancer cell line panel we profiled global DNA methylation patterns across the genome in the presence and absence of DNMT3B. Upon DNMT3B removal there was a statistically significant loss of methylation in Gene Bodies, 3' UTRs, Shores, and Shelves. Interestingly, loss of DNMT3B had a greater effect on the methylation status of non-CpG island genes when compared to CpG island genes. Re-introduction of wild-type DNMT3B resulted in a gain of methylation in these areas. Unexpectedly, mutant DNMT3B also caused increased methylation, suggesting DNMT3A induction. This data supports the notion DNMT3B regulates DNA methylation in a context-dependent manner at distinct regions of the human genome.

Local studies identified endogenous target genes and demonstrated that DNMT3B is capable of modulating gene expression independently of its methyltransferase activity.

Both wild-type and catalytically dead DNMT3B repressed target genes to similar degrees, suggestive of protein-protein interactions at the N-terminus. A DNMT3B-specific loss of methylation may help to explain expression changes seen in non-CpG island genes. However, it was not required for gene repression at CpG island genes. Further studies in genetic knockout cells alluded to changes in chromatin status upon DNMT3B overexpression that explained transcriptional repression at this subset of genes, such as decreased enrichment for a histone modification associated with transcriptional activity (H3K4me3) and the recruitment of a repressive epigenetic protein (LSD1).

First Reader/Advisor: Stephen B. Baylin, M.D.

Second Reader: Robert A. Casero, Jr. , Ph.D.

Acknowledgements

First, I am forever indebted to Dr. Stephen B. Baylin for the opportunity to become a part of the Baylin lab lineage. Under Steve's mentorship and tutelage, I have learned so much about epigenetics and grown to be an independent scientist. I will carry these invaluable lessons with me through my academic career. A special expression of gratitude to my thesis committee: Dr. Robert A. Casero, Jr., Dr. James Stivers, Dr. Ben Ho Park, and Dr. Hongjun Song for all of their guidance. I would like to especially thank Dr. James Herman and my Baylin/Herman labmates for all of their support and smiles over the years. In particular, I would like to show heartfelt appreciation to Andre, Christin, Eriko, Hari, Heather, Helai, Joo Mi, Kathy, Ray-Whay, Sen, Tina, Wei, and Yi. I'll never forget your friendship. When science was tough, you reminded me I was tougher.

I would be remiss if I did not thank my support systems outside of lab. In particular, I'd like to thank Dr. David Valle, Dr. Kirby Smith, and Sandy for helping me live my dream of earning my PhD in the Pre-doctoral Training Program in Human Genetics. I selected this program during my first year of college and am so proud to carry on its great legacy. To my dearest friends: April, Audrey, Ceora, Chevron, Gerard, Justin, Leilani, Maria, Nanlesta, Natasha, Nina, Sika, Tyesha, and Yolanda, thank you for always being there for me. I'm looking forward to witnessing your future endeavors and milestones. To all of my Junior Research Scholars mentees, I will be there to watch you get hooded one day. Finally, I would like to thank the Tinsley, Sanders, Robinson, and Mitchell families. Every call, text, e-mail, hug, and prayer was appreciated. This thesis is dedicated to Carol, Carla, and Louise ... my life's inspiration.

Table of Contents

Title	i
Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Tables	vii
List of Figures	viii
Chapter 1 - Introduction	1
The definition of epigenetics	2
Biological significance of DNA methylation	2
DNA methylation and cancer	3
Mammalian DNA methyltransferases	4
DNA methyltransferases, transcription factors, and transcriptional repression	5
DNA methyltransferases, chromatin modifications, and transcriptional repression	6
Functions of DNMT3B in Development and Disease	8
Thesis goal and specific aims	9
Chapter 2 - DNMT3B regulates DNA methylation in a context-dependent manner at distinct regions of the human genome.	20
Abstract	21
Introduction	22
Materials and Methods	24
Results	27
Discussion	47

Acknowledgements	49
References	50
Chapter 3 - DNMT3B (a de novo DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity.	54
Abstract	55
Introduction	57
Materials and Methods	59
Results	62
Discussion	89
Acknowledgements	90
References	91
Chapter 4 - Conclusions and Future Directions	93
References	98
Curriculum Vitae	100

List of Tables

3.1	qRT-PCR Primer Sequences	88
3.2	ChIP Primer Sequences	88

List of Figures

1.1.	DNA methylation patterns in normal and cancer cells.	12
1.2.	DNA methyltransferase protein domains.	13
2.1.	Transient transfection of wild-type (WT) and mutant (MUT) DNMT3B into <i>DNMT3B</i> ^{-/-} cells.	31
2.2.	The Infinium HumanMethylation450 BeadChip Array Platform.	32
2.3.	Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location.	33-40
2.4.	Paired comparison of hypermethylation in parental HCT116 relative to <i>DNMT3B</i> ^{-/-} Cells.	41
2.5.	Paired Comparison of Hypermethylated Probes in Parental HCT116 (β values ≥ 0.50) to hypomethylated probes in <i>DNMT3B</i> ^{-/-} Cells (β values ≤ 0.2).	42-43
2.6.	Identification of genes affected by loss of DNMT3B.	44
2.7.	Expression status of genes that correspond with increased probe methylation.	45-46
3.1.	Statistical filtering of DNMT3B candidate genes.	69
3.2.	Genetic disruption of DNMT3B in parental HCT116.	70
3.3.	Pharmacologic inhibition of DNMTs in parental HCT116 and 3BKO cells.	71
3.4.	The effect of wild type and mutant DNMT3B on target gene expression.	72
3.5.	Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location.	73-80
3.6.	Paired comparison of methylation in parental HCT116 relative to 3BKO cells.	81
3.7.	Methylation patterns according to CpG status at promoters.	82
3.8.	Methylation patterns according to CpG status at gene bodies.	83

3.9.	Representative plots of methylation across DNMT3B target genes.	84
3.10.	A methylation comparison between non-CpG island DNMT3B targets and random genes.	85
3.11.	Repressive protein recruitment to DNMT3B target genes.	86
3.12.	Histone modifications at DNMT3B target genes.	87

Chapter 1

Introduction

The definition of epigenetics

Conrad Hal Waddington first introduced the term "epigenetics" in a series of seminal work geared towards constructing an integrated view of biology. Waddington's definition stated "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" [1]. As our understanding of epigenetics evolved, so did the meaning of the word. Our modern definition of epigenetics is the study of heritable changes (mitotic or meiotic) in gene function without a corresponding change in DNA sequence [2, 3]. Epigenetic mechanisms can be covalent or noncovalent alterations to DNA, RNA [4], or proteins [5, 6]; including DNA methylation, histone modifications, non-coding RNA interactions, and changes in chromatin structure or architecture. One of the most well-characterized epigenetic mechanisms is DNA methylation, which is both a heritable and reversible process.

Biological significance of DNA methylation

DNA methylation is a covalent biochemical modification that plays important biological roles in a variety of species, including fungi, plants, vertebrates, and humans. It occurs at the carbon 5 position of the cytosine ring. The multi-step enzymatic mechanism first involves binding to DNA and flipping the target sequence out of the double helix. Next, a transient covalent complex is formed. Finally, there is a transfer of the methyl group from the donor S-Adenosyl Methionine (SAM) to recipient DNA [7].

Proper DNA methylation patterning is required for many different biological processes, such as development [8], differentiation [8], regulation of gene expression [9], X-chromosome inactivation [10], genomic imprinting [11], and silencing of exogenous

retroviral DNA [12]. For example, DNA methylation is extensively reprogrammed during embryonic development. The paternal genome is actively demethylated soon after fertilization; while the maternal genome is passively demethylated after a failure to maintain methylation through early rounds of successive cell division. Cellular fate can also be determined by DNA methylation through the targeting of specific genes important for pluripotency and differentiation. Most importantly for this work, the presence of DNA methylation can regulate gene expression by strongly interfering with the ability of transcription factors to bind the promoter [13]. It is growing increasingly clear that dysregulation of DNA methylation plays an important role in the etiology of numerous diseases. Much of our understanding stems from knowledge of DNA methylation dynamics in cancer.

DNA methylation and cancer

DNA methylation patterns are not randomly distributed throughout the genome. Instead they are compartmentalized by region. Heterochromatin, which contains centromeres, telomeres, and repetitive DNA sequences, tends to be hypermethylated and transcriptionally silent in normal cells. The rest of the genome is predominantly CpG-poor and devoid of methylation. However, there are some CpG-rich regions. Such regions are found at promoters and contain a dense concentration of CpG dinucleotide clusters called CpG islands. Normally, DNA methylation is rare at CpG islands. These genomic features tend to be hypomethylated and associated with active genes.

In tumors, the normal compartmentalization is reversed. Aberrant DNA methylation patterns are frequently seen in cancer, both on a genome-wide scale and at specific genes [9]. Cancer cells are characterized by global hypomethylation, that

primarily affects repetitive DNA and leads to genomic instability, with concurrent site-specific CpG island hypermethylation (Figure 1.1). Hypermethylation of tumor suppressor genes in the promoter region often leads to gene silencing. Since epigenetic events are reversible, these abnormal alterations are rich therapeutic targets. DNA methylation is currently being used as a tool for better cancer diagnosis, prognosis, and prediction to therapeutic response in the clinic [14]. A crucial step towards a better understanding of tumorigenesis is to demystify how tumors both acquire and maintain abnormal methylation patterns. These processes are mediated by DNA methyltransferases (DNMTs).

Mammalian DNA methyltransferases

DNMTs are a family of enzymes that transfer a methyl group from SAM to the carbon 5 position of cytosine residues in DNA, which occur in CpG dinucleotides. Only 3 out of 5 are enzymatically active. From a classical perspective, the active mammalian DNMTs have been assumed to fall into 2 broad functional categories, maintenance and *de novo*. DNMT1 has a preference for hemimethylated DNA (5-30%) and is localized to DNA replication [15]. Consequently, it is assigned the function of maintenance methylation. DNMT3A and DNMT3B show no preference for hemimethylated DNA [16] and are thought to establish new methylation patterns [17]. DNMT3L lacks a catalytic domain but complexes with and stimulates the activity of DNMT3A and DNMT3B [18, 19]. DNMT2 contains all of the conserved motifs shared by the other known DNMTs, but it does not perturb *de novo* or maintenance methylation. Instead, it is responsible for methylating a small RNA (aspartic acid tRNA) [20]. Misregulation of DNMTs have been attributed to tumorigenesis. For example, elevated Dnmt3b1

expression in the $Apc^{Min/+}$ mouse model has been shown to enhance colorectal carcinogenesis and cause methylation of tumor suppressor genes, leading to their silencing [21].

Structurally, the enzymatically active DNMTs (DNMT1, DNMT3A, and DNMT3B) can be divided into 2 domains (Figure 1.2). The C-terminus of all three proteins contain a highly conserved catalytic motif, but the N-terminal region is not highly conserved. However, paralogous proteins DNMT3A and DNMT3B share a similar feature at the N-terminus. The PHD domain, also referred to as the ATRX-like domain, has been implicated in protein-protein interactions and is a common motif found in chromatin-associated proteins [22].

DNA methyltransferases, transcription factors, and transcriptional repression

Mammalian gene expression is a complex process involving both transcriptional activators as well as repressors. The impact of genetic alterations on gene regulation has been well studied. As a field, epigenetics is adding to our repertoire of factors that control gene expression. DNA methylation at promoters functions to repress transcription and DNMTs are key epigenetic regulatory proteins implicated in this molecular mechanism.

Interactions between DNMTs and transcription factors have been proposed to target specific promoters for methylation. Thus, both overlapping and distinct DNMT targeting can be expected. DNMT3B, but not DNMT1, has been shown to regulate specific genes in human cancers [23-25]. Although DNA methylation prevents their promoter binding, some transcription factors are known to recruit DNMT3B [26, 27] or directly interact with the protein [4, 28]. This evidence suggests DNMT3B has distinct endogenous targets on which it exerts its transcriptional regulatory effects.

DNA methyltransferases, chromatin modifications, and transcriptional repression

Chromatin is the complex of DNA and histone proteins that act as a molecular scaffold for the packaging of our genome. Histones are a family of basic proteins which wrap around negatively charged DNA. H1 is a non-core histone that interacts with linker DNA between histones. Other histones are found in octamer cores containing two copies each of H2A, H2B, H3, and H4. Histone methylation occurs at lysine (K) and arginine (R) residues of N-terminal tails that extend from the histone core. They may be mono-, di-, or trimethylated, with variable functionality according to the number of methyl groups.

Many studies have shown when the trimethylation of lysine 4 in the histone H3 core (H3K4me3) is localized to promoter regions it correlates with transcriptional activation [29]. Conversely, the methylation of other histone marks have been associated with transcriptional repression. H3K9me3 is associated with transcriptional silencing and heterochromatin formation [30]. Clearly, histone methylation is a context-dependent modification that promotes variable transcriptional effects. Unlike methylation, histone acetylation is obligately linked to transcriptional activation and can be found both on the N-terminal tails and histone core. Acetyl groups add negative charge to positively charged lysines, thus reducing the interactions between DNA and histones. This opening of the tightly packed nucleosome allows transcriptional machinery access to the DNA and facilitates gene transcription.

Polycomb repressive complexes (PRC) remodel chromatin and can also contribute to a repressive chromatin state. EZH2, a catalytic component of PRC2, can catalyze trimethylation of lysine 27 of histone H3 (H3K27me3) [31]. H3K27me3 can

also recruit PRC1, the maintenance complex. How PRCs are recruited to gene targets remain unclear. Some work suggests the involvement of DNMT3B. DNMT3B1 has been associated with genes that harbor repressive H3K27me3 modifications and transcriptional inactivity [32]. Other studies show Dnmt3b preferentially interacts with nucleosomal DNA and higher-ordered chromatin as opposed to naked DNA [33, 34]. Dnmt3b, but not Dnmt3a, associated with linker histone H1 without enrichment of silent chromatin marks [34]. These results suggest Dnmts differentially interact with higher order chromatin.

Dynamic cross talk between DNA methylation and histone modifications occur in different cellular contexts. Some evidence suggests genomic methylation depends on histone methylation. In *Neurospora crassa*, H3K9 replacement and removal of the H3K9 methyltransferase (DIM5) resulted in a loss of DNA methylation [35]. In mouse ES cells, H3K9 methyltransferase deficiency (Suv39h1 and Suv39h2) correlates with hypomethylation at repeat elements [36]. In addition, Suv39h has been shown to direct H3K9me3 at pericentromeric repeats, regions known to be controlled by Dnmt3b. G9a, another H3K9 methyltransferase, can promote DNA methylation independently of its enzymatic activity [37], possibly through interactions with DNMT1 [38]. Presumably, DNA methylation acts downstream from repressive marks like H3K9 methylation.

Chromatin structure can also regulate transcription by controlling what DNA-binding factors have access to chromatin [39-41]. In addition to histone modifications, nucleosome positioning and turnover, and chromatin remodeling factors all determine chromatin structure. The cumulative effect of the DNA methylation and chromatin changes influence the transition from a repressed, inactive state to an active state that permits transcription.

Functions of DNMT3B in Development and Disease

The strongest evidence supporting the biological importance of DNMT3s in establishing DNA methylation patterns comes from studies of early mouse development. After fertilization zygotes undergo active demethylation of the paternal genome, followed by genome-wide remethylation carried out by *de novo* methyltransferases [42, 43]. Work in single mutant ES cells lacking Dnmt3a or Dnmt3b were still able to actively methylate proviral DNA after a new retroviral infection. However, double mutant ES cells were completely devoid of *de novo* methylation activity, suggesting both Dnmt3a and Dnmt3b are functionally redundant and at least one is required to establish new methylation patterns [17].

While Dnmt3a^{-/-} mice developed to term and appeared normal at birth, they succumbed by 4 weeks of age. By contrast, Dnmt3b^{-/-} mice were unviable at birth. It appears the DNMT3 family is essential for early mammalian development [17]. The fact that Dnmt3a^{-/-} and Dnmt3b^{-/-} mice display a different developmental phenotype suggests the two proteins may also have distinct functional properties. This notion is supported by ICF syndrome, a rare human disease characterized by DNMT3B deficiency [44].

ICF is the only human disease attributed to a genetic mutation in a DNA methyltransferase. With the loss of DNMT3B, non-coding repetitive sequences and genes located in constitutive and facultative heterochromatin are routinely hypomethylated in ICF cells [45]. This hypomethylation is associated with chromatin decondensation and chromosomal instability [45], similar phenotypes seen in cancer.

Thesis goal and specific aims

Dnmt3b has been shown to be downregulated upon differentiation and expressed at low levels in adult somatic tissues [17]. Several studies have reported either overexpression or variant expression of DNMT3B in a variety of cancers, suggesting a role for the protein in tumorigenesis [46-48]. Contrary to its canonical *de novo* methylation role, a series of experiments using DNMT genetic knockout cell lines suggest DNMT1 and DNMT3B cooperate to maintain methylation in human cancers [49]. This study focused on how DNMT3B-specific methylation changes correlated with gene expression and/or chromatin changes.

Our lab was amongst the first to show the N-terminal domains of Dnmt3a and Dnmt3b alone can repress transcription of exogenous reporter genes [50]. This domain specific repression is partially mediated by HDACs, histone modifying enzymes known to promote transcriptional inactivity [50, 51]. One drawback to such studies is the inability to capture how truncated proteins change 3-dimensional structure, and by extension the potential disruption of important protein-protein interactions which may concurrently play a role in gene regulation. Also, reporter gene activity may not accurately reflect what happens in an endogenous setting due to a lack of other regulatory factors normally present in the cell.

This work sought to shed light on how DNMT3B-mediated DNA methylation targets methylation both genome-wide and at specific genes. Furthermore, we looked at the protein's role in regulating specific genes independently from its methyltransferase activity. We address shortcomings in previous studies by introducing a point mutation in a key residue critical for catalysis, which maintained the structural integrity of the

DNMT3B protein but also abolished all catalytic activity. Additionally, analyses were done exclusively on endogenous DNMT3B target genes.

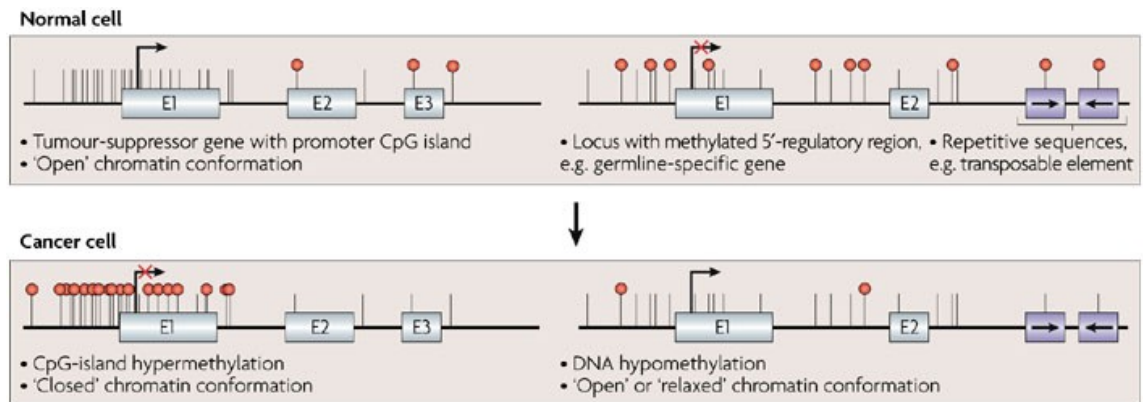
Aim 1: Identify differentially methylated genomic regions controlled by DNMT3B.

O'Hagan *et al* has shown DNMT3B complexes with DNMT1 and PRC4 upon oxidative damage. Interestingly, this complex relocalized from nonCpG-rich regions to CpG island promoters upon hydrogen peroxide treatment [52]. High expression genes were characterized by changes in histone marks and nascent transcription, while low expression genes gained DNA methylation. In another report, the genetic deletion of DNMT3B resulted in a 3% decrease in global methylation [49]. From our understanding of DNMT3B biology, it can be expected these changes occur at repetitive DNA sequences. However, this has never been confirmed. Technical limitations prevented deeper probing as to what specific genomic regions experienced this subtle loss. Both studies raise interesting questions about where DNMT3B is normally localized, its cancer-specific localization, and protein's role in initiating or maintaining methylation patterns across genomic regions. We used an Infinium 450K methylation array to analyze and further refine genome wide methylation patterns in cells both lacking and possessing DNMT3B.

Aim 2: Elucidate mechanisms responsible for gene repression at DNMT3B gene targets.

Recent work from our group has shown DNMT1 can modulate gene expression without its catalytic activity, partially through its interactions with histone-modifying enzymes [53]. Clements *et al.* identified endogenous DNMT1 target genes and proved the protein represses target genes independent of its catalytic activity. Interestingly, DNA

methylation was not required for gene repression of the identified targets. When querying the chromatin status, active chromatin marks were depleted in the presence of either a wild type or mutant DNMT1 after recruitment to gene promoters. Repressive chromatin marks remained unchanged with the loss of DNMT1 alone at the target genes. By contrast, little is known about discrete genes under the exclusive control of DNMT3B. We identified endogenous targets and assessed their methylation and chromatin status to determine the regulatory effect of DNMT3B on gene expression.

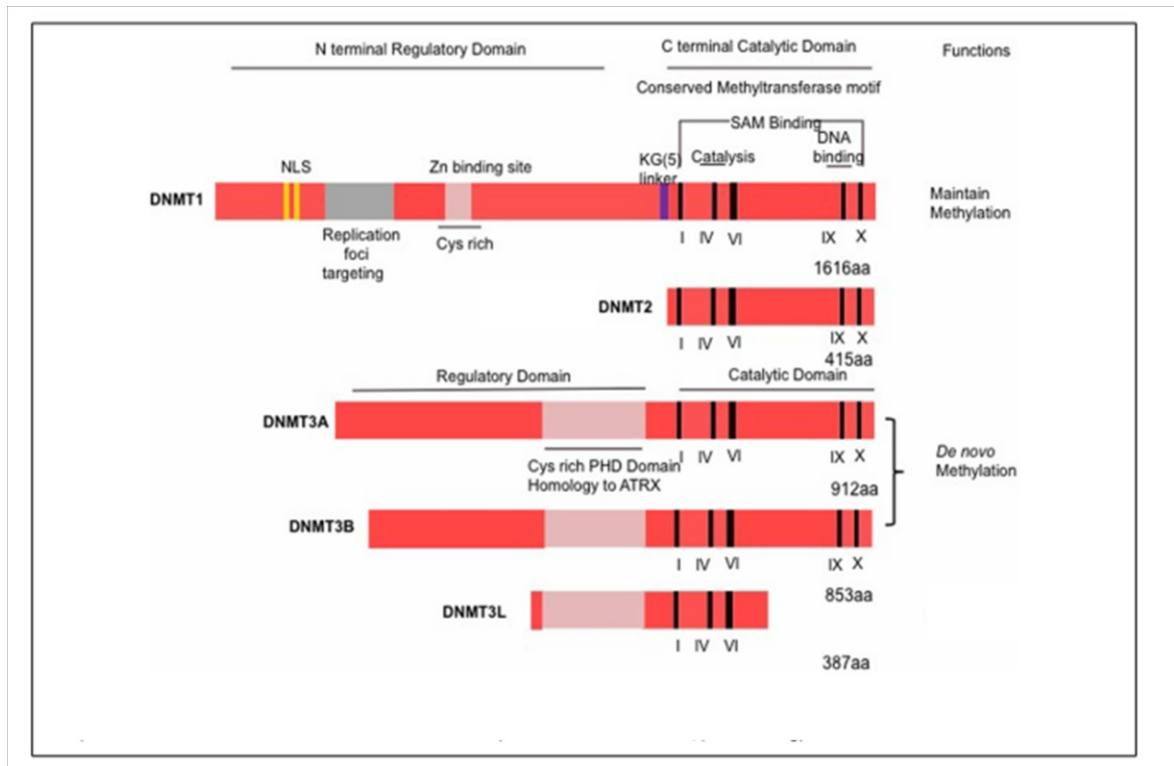


Esteller, M., *Cancer epigenomics: DNA methylomes and histone-modification maps*.

Nature Reviews Genetics, 2007. 8(4): p. 286-298.

Figure 1.1. DNA methylation patterns in normal and cancer cells. (Top panel)

Normal cells are characterized by hypomethylated CpG island promoters at tumor suppressor genes. Exceptions include, germline-specific, tissue-specific, and imprinted genes which can possess extensive amounts of promoter methylation. Repetitive sequences are highly methylated to prevent active transcription. (Bottom panel) Cancer cells have aberrant DNA methylation patterns. CpG island promoters gain hypermethylation and the rest of the genome is globally hypomethylated, including previously hypermethylated gene regulatory regions and repetitive sequences. Hypomethylated repetitive sequences in cancer lead to the characteristic genomic instability seen in tumors.



Adapted from: Subramaniam, D., *DNA methyltransferases: A novel target for prevention and therapy*. Frontiers in Oncology, 2014. 4(80): p. 1-13.

Figure 1.2. DNA methyltransferase protein domains. The N-terminus contains domains that interact with DNA and facilitate protein-protein interactions. The C-terminal domain contains the conserved catalytic motif responsible for methyltransferase activity.

References

1. Waddington, C., *The epigenotype*. Endeavour, 1942. **1**: p. 18-20.
2. Goldberg, A.D., C.D. Allis, and E. Bernstein, *Epigenetics: a landscape takes shape*. Cell, 2007. **128**(4): p. 635-8.
3. Bird, A., *Perceptions of epigenetics*. Nature, 2007. **447**(7143): p. 396-8.
4. He, C., *Grand challenge commentary: RNA epigenetics?* Nat Chem Biol, 2010. **6**(12): p. 863-5.
5. Jenuwein, T. and C.D. Allis, *Translating the histone code*. Science, 2001. **293**(5532): p. 1074-80.
6. Berger, S.L., *The complex language of chromatin regulation during transcription*. Nature, 2007. **447**(7143): p. 407-12.
7. Bestor, T.H., *The DNA methyltransferases of mammals*. Hum Mol Genet, 2000. **9**(16): p. 2395-402.
8. Goll, M.G. and T.H. Bestor, *Eukaryotic cytosine methyltransferases*. Annu Rev Biochem, 2005. **74**: p. 481-514.
9. Baylin, S.B. and P.A. Jones, *A decade of exploring the cancer epigenome - biological and translational implications*. Nat Rev Cancer, 2011. **11**(10): p. 726-34.
10. Heard, E., P. Clerc, and P. Avner, *X-chromosome inactivation in mammals*. Annu Rev Genet, 1997. **31**: p. 571-610.
11. Li, E., C. Beard, and R. Jaenisch, *Role for DNA methylation in genomic imprinting*. Nature, 1993. **366**(6453): p. 362-5.

12. Smith, Z.D. and A. Meissner, *DNA methylation: roles in mammalian development*. Nat Rev Genet, 2013. **14**(3): p. 204-20.
13. Jones, P.A. and S.B. Baylin, *The epigenomics of cancer*. Cell, 2007. **128**(4): p. 683-92.
14. Heyn, H. and M. Esteller, *DNA methylation profiling in the clinic: applications and challenges*. Nat Rev Genet, 2012. **13**(10): p. 679-92.
15. Leonhardt, H., et al., *A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei*. Cell, 1992. **71**(5): p. 865-73.
16. Okano, M., S. Xie, and E. Li, *Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases*. Nat Genet, 1998. **19**(3): p. 219-20.
17. Okano, M., et al., *DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development*. Cell, 1999. **99**(3): p. 247-57.
18. Chedin, F., M.R. Lieber, and C.L. Hsieh, *The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a*. Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16916-21.
19. Ooi, S.K., et al., *DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA*. Nature, 2007. **448**(7154): p. 714-7.
20. Goll, M.G., et al., *Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2*. Science, 2006. **311**(5759): p. 395-8.
21. Linhart, H.G., et al., *Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing*. Genes Dev, 2007. **21**(23): p. 3110-22.

22. Aasland, R., T.J. Gibson, and A.F. Stewart, *The PHD finger: implications for chromatin-mediated transcriptional regulation*. Trends Biochem Sci, 1995. **20**(2): p. 56-9.
23. Yun, J., et al., *Gene silencing of EREG mediated by DNA methylation and histone modification in human gastric cancers*. Lab Invest, 2012. **92**(7): p. 1033-44.
24. Fan, H., et al., *MTSS1, a novel target of DNA methyltransferase 3B, functions as a tumor suppressor in hepatocellular carcinoma*. Oncogene, 2012. **31**(18): p. 2298-308.
25. Hlady, R.A., et al., *Loss of Dnmt3b function upregulates the tumor modifier Ment and accelerates mouse lymphomagenesis*. J Clin Invest, 2012. **122**(1): p. 163-77.
26. Hashimoto, H., et al., *Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation*. Nucleic Acids Res, 2012. **40**(11): p. 4841-9.
27. Velasco, G., et al., *Dnmt3b recruitment through E2F6 transcriptional repressor mediates germ-line gene silencing in murine somatic tissues*. Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9281-6.
28. Hervouet, E., F.M. Vallette, and P.F. Cartron, *Dnmt3/transcription factor interactions as crucial players in targeted DNA methylation*. Epigenetics, 2009. **4**(7): p. 487-99.
29. Feng, W., et al., *PHF8 activates transcription of rRNA genes through H3K4me3 binding and H3K9me1/2 demethylation*. Nat Struct Mol Biol, 2010. **17**(4): p. 445-50.

30. Lachner, M., et al., *Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins*. Nature, 2001. **410**(6824): p. 116-20.
31. Guil, S., et al., *Intronic RNAs mediate EZH2 regulation of epigenetic targets*. Nat Struct Mol Biol, 2012. **19**(7): p. 664-70.
32. Choi, S.H., et al., *Identification of preferential target sites for human DNA methyltransferases*. Nucleic Acids Res, 2011. **39**(1): p. 104-18.
33. Shen, L., et al., *A single amino acid substitution confers enhanced methylation activity of mammalian Dnmt3b on chromatin DNA*. Nucleic Acids Res, 2010. **38**(18): p. 6054-64.
34. Kashiwagi, K., et al., *DNA methyltransferase 3b preferentially associates with condensed chromatin*. Nucleic Acids Res, 2011. **39**(3): p. 874-88.
35. Tamaru, H. and E.U. Selker, *A histone H3 methyltransferase controls DNA methylation in Neurospora crassa*. Nature, 2001. **414**(6861): p. 277-83.
36. Lehnertz, B., et al., *Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin*. Curr Biol, 2003. **13**(14): p. 1192-200.
37. Dong, K.B., et al., *DNA methylation in ES cells requires the lysine methyltransferase G9a but not its catalytic activity*. EMBO J, 2008. **27**(20): p. 2691-701.
38. Esteve, P.O., et al., *Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication*. Genes Dev, 2006. **20**(22): p. 3089-103.

39. Cairns, B.R., *The logic of chromatin architecture and remodelling at promoters.* Nature, 2009. **461**(7261): p. 193-8.
40. Clapier, C.R. and B.R. Cairns, *The biology of chromatin remodeling complexes.* Annu Rev Biochem, 2009. **78**: p. 273-304.
41. Fuda, N.J., M.B. Ardehali, and J.T. Lis, *Defining mechanisms that regulate RNA polymerase II transcription in vivo.* Nature, 2009. **461**(7261): p. 186-92.
42. Mayer, W., et al., *Demethylation of the zygotic paternal genome.* Nature, 2000. **403**(6769): p. 501-2.
43. Oswald, J., et al., *Active demethylation of the paternal genome in the mouse zygote.* Curr Biol, 2000. **10**(8): p. 475-8.
44. Xu, G.L., et al., *Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene.* Nature, 1999. **402**(6758): p. 187-91.
45. Lana, E., et al., *DNA replication is altered in Immunodeficiency Centromeric instability Facial anomalies (ICF) cells carrying DNMT3B mutations.* Eur J Hum Genet, 2012. **20**(10): p. 1044-50.
46. Saito, Y., et al., *Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 10060-5.
47. Ostler, K.R., et al., *Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins.* Oncogene, 2007. **26**(38): p. 5553-63.

48. Gopalakrishnan, S., et al., *A novel DNMT3B splice variant expressed in tumor and pluripotent cells modulates genomic DNA methylation patterns and displays altered DNA binding*. Mol Cancer Res, 2009. **7**(10): p. 1622-34.
49. Rhee, I., et al., *DNMT1 and DNMT3b cooperate to silence genes in human cancer cells*. Nature, 2002. **416**(6880): p. 552-6.
50. Bachman, K.E., M.R. Rountree, and S.B. Baylin, *Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin*. J Biol Chem, 2001. **276**(34): p. 32282-7.
51. Bai, S., et al., *DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2*. Mol Cell Biol, 2005. **25**(2): p. 751-66.
52. O'Hagan, H.M., et al., *Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG Islands*. Cancer Cell, 2011. **20**(5): p. 606-19.
53. Clements, E.G., et al., *DNMT1 modulates gene expression without its catalytic activity partially through its interactions with histone-modifying enzymes*. Nucleic Acids Res, 2012. **40**(10): p. 4334-46.

Chapter 2

DNMT3B regulates DNA methylation in a context-dependent manner at distinct regions of the human genome.

Abstract

DNA methylation is a well-studied epigenetic modification important for normal development and dysregulated in a variety of diseases. Cancer is characterized by global hypomethylation and hypermethylation of tumor suppressor genes. DNA methylation dynamics are regulated by the DNMTs (DNMT1, DNMT3A, and DNMT3B). The *de novo* methyltransferase, DNMT3B, has been implicated in the etiology of various diseases, including cancer. Classic studies suggested DNMT3B only localized to gene promoters. However, recent reports show it can also bind to gene bodies. Profiling DNA methylation patterns across the genome in the presence and absence of DNMT3B will provide novel insights into the functional role of this protein. In this study, an isogenic colorectal cancer cell line panel was subjected to an epigenome-wide scan using the Infinium HumanMethylation450 BeadChip platform, followed by gene expression analysis.

When DNMT3B is removed there is a loss of methylation across the genome, with the greatest change occurring in Gene Bodies, 3' UTRs, Shores, and Shelves. These methylation changes are subtle, yet significant (according to two-tailed paired t-tests). Interestingly, loss of DNMT3B has a greater effect on the methylation status of non-CpG island genes than CpG island containing genes. Expression changes displayed a bimodal distribution of correlation with methylation status at both promoters and gene bodies. Suggesting DNMT3B may work in concert with other proteins at discrete genomic regions to elicit transcriptional changes. Collectively, these data represent novel insights into the potential regulatory role of DNMT3B on DNA methylation at non-promoter associated regions of the genome.

Introduction

DNA methylation is one of the most well-studied epigenetic modifications [1]. It is responsible for normal development in a variety of species and dysregulated in many diseases [2]. Cancer cells are characterized by global hypomethylation with region-specific hypermethylation at CpG island promoters of particular genes [3]. Most studies have focused on how DNA methylation at gene promoters function to repress transcription [4]. By contrast, the relationship of CpG methylation at other genomic regions to transcription is less well understood.

Definitively elucidating the function of gene body methylation is an area of strong interest and active investigation [5, 6]. Gene body methylation is widespread in eukaryotes and has been implicated in transcriptional elongation, silencing of repetitive DNA in intragenic regions, regulating alternative splicing and alternative promoter usage, as well as regulating insulators and enhancers [6]. The exact functional consequences warrant further study. Genome-wide methylation and expression data suggest gene body methylation is evolutionarily conserved and correlated with transcriptional activity [5, 7]. Mechanistically, a DNMT3 family member has been shown to antagonize Polycomb repression at gene bodies and promote an active chromatin state [8].

Interestingly, DNA methylation in honeybees occurs primarily at gene bodies [9, 10] and is regulated by DNA methyltransferase 3 (*dnmt3*) [11, 12], the orthologous protein to the *de novo* DNA methyltransferase 3B (DNMT3B) in humans. In cells from patients with immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome, a disease characterized by *DNMT3B* mutations, hypermethylation of active gene bodies is severely disrupted [13-15]. In human cancers, DNMT3B has been shown

to function as a transcriptional repressor [16-20] and may be responsible for the activation of tissue-specific programs that lead to early tumor formation [21].

We used an isogenic colorectal cancer cell line model genetically devoid of DNMT3B [22] to test the hypothesis that DNMT3B may regulate DNA methylation at non-promoter regions of the human genome. This study is the first of its kind to investigate the functional consequences of the removal and re-introduction of both wild type and mutant DNMT3B on global methylation patterns and gene expression in human cancer.

Materials and Methods

Cell culture

HCT116, *DNMT1*^{-/-} hypomorph subclone 5F (MT1KO), *DNMT3B*^{-/-} subclone 2 (3BKO), and *DNMT1*^{-/-} hypomorph; *DNMT3B*^{-/-} subclone 2 (DKO) were previously described [22, 23]. All cell lines were cultured in McCoy's 5A (Iwakata & Grace Modification) (Cat No. 10-050-CV, Corning cellgro) supplemented with 10% fetal bovine serum (Cat No. 35-011-CV, Corning cellgro) at 37 °C and 5% CO₂ atmosphere.

Plasmid constructs

Wild type *DNMT3B* Isoform 1 (NM_006892.3) with N-terminal HA tag inserts were subcloned into a pEF1α IRES-puro vector (WT3B). A QuikChange II Site-Directed Mutagenesis Kit (Cat No. 200523, Agilent) was used to generate a catalytically inactive *DNMT3B* (C651W) (MUT3B).

Transient transfection

The *DNMT3B*^{-/-} subclone 2 (3BKO) cell line was transiently transfected for 48 hours with 1 µg of both wild type and mutant plasmid DNA using Lipofectamine 2000 (Cat No. 11668-027, Life Technologies).

Western blot analysis

Nuclear isolations were performed by resuspending cell pellets in CEBN with protein inhibitors and incubating on ice for 10 minutes, while vortexing every minute. After centrifugation the supernatant containing cytoplasmic fraction was saved for later use and nuclear pellets were washed in CEB with protein inhibitors. Nuclear pellets were resuspended in modified RIPA buffer with protein inhibitors, spermine, and spermidine,

followed by sonication and BCA assay. Anti β -actin (Cat No. A5441, Sigma-Aldrich) and anti HA-HRP (Cat No. H6533, Sigma-Aldrich) antibodies were used for analysis.

Genomic DNA isolation

DNeasy Blood and Tissue Kit™ (Cat No. 69504, Qiagen) was used to extract 1 μ g genomic DNA from each cell line according to manufacturer's protocol. Isolated genomic DNA was bisulfite converted using EZ-DNA Methylation™ Kit (Cat No. D5001, Zymo Research).

Methylation analysis with the Infinium HumanMethylation450 BeadChip Kit

DNA methylation analyses of the 9 experimental samples (biological triplicates of 3BKO, 3BKO overexpressing WT3B, and overexpressing MUT3B) and 3 control cell lines (HCT116, MT1KO, and DKO) were performed with the Infinium HumanMethylation450 BeadChip Kit (Cat. No. WG-314-1001, Illumina) according to the manufacturer's protocols. The platform contains 486,428 CpG sites distributed across the promoter, 5' UTR, TSS, first exon, gene body, 3' UTR, CpG island, shore, and shelf regions and covers 99% of RefSeq genes [24].

Methylation data processing and visualization

Illumina GenomeStudio Methylation Module software (Part No. 11319130 Rev. B, Illumina) was used to calculate methylation levels and identify methylation signatures across the entire genome. Reported β -values reflect the estimated methylation level of each CpG locus using the ratio of intensities between methylated and unmethylated probes. The Bioconductor package in the R programming and software environment was used to further analyze and visualize methylation data.

RNA isolation

RNeasy Mini Kit™ (Cat No. 74104, Qiagen) was used to extract 1 µg total RNA from each cell line according to manufacturer's protocol. Isolated total RNA was treated with RNase-Free DNase I (Cat No. 79254, Qiagen).

Gene expression analysis with human gene expression microarrays

Total RNA was hybridized to Human GE 4x44K v2 Microarray (Cat. No. G4845A, Agilent) according to manufacturer's protocol.

Expression data processing and visualization

The Bioconductor and Limma packages in the R programming and software environment were used to further analyze and visualize expression data. Log₂ ratios were calculated. Genes with a Log₂ ratio of ≥ 0.5 were described as upregulated and ≤ -0.5 were considered downregulated.

Results

Global DNA methylation patterns upon DNMT3B removal and re-introduction

We first tested DNA constructs to ensure robust overexpression of both wild type and mutant DNMT3B. A point mutation was introduced into the C-terminal domain of DNMT3B, causing a Cysteine to Tryptophan substitution at residue 651. This residue is critical for catalysis and abolishes all catalytic activity without a change in 3-dimensional structure [25]. Western blot analysis indicates cells express the two exogenous DNMT3B species to similar degrees, independent of DNMT3B mutation status (Figure 2.1).

Next, Infinium 450K arrays were used as an epigenome-wide scan to assess the methylation status of various genomic signatures (TSS, 5' UTR, Promoter, Gene Body, 3' UTR, CpG Island, Shore, and Shelf) (Figure 2.2). Parental HCT116 is the most hypermethylated (β values ≥ 0.5) cell line used in this study and DKO is the least methylation (β values ≤ 0.5). Kernel density plots of the methylation data revealed changes in "promoter-associated" regions were minimal (Figure 2.3 A-C, F). In TSS (TSS1500 and TSS200), 5' UTR, Promoter (manually defined as CpGs found in -500 to +1500 with respect to the TSS), and CpG Island regions most β values failed to surpass a density of 4.0 in HCT116, reflecting little methylation. Most of the β values were ≤ 0.2 . Low basal methylation in the parental line explains the failure to detect appreciable loss of methylation at these "promoter-associated" signatures in the 3BKO cells.

Comparatively, "non-promoter associated" regions like Gene Body and 3' UTR regions were more densely methylated in HCT116 and displayed decreased methylation in the absence of DNMT3B (Figure 2.3 D-E). Shores and shelves are flanking regions a significant distance away from CpG Islands, 2 kb and 4 kb respectively (Figure 2.2).

These regions also appeared to lose DNA methylation (Figure 2.3 G-H). Thus, DNA methylation appears to be more prominent and readily lost in the areas where transcription is not usually initiated (Gene Body, 3' UTR, Shore, and Shelf regions). Overexpression of both the wild type and mutant DNMT3B caused a gain of methylation in these regions. This was surprising since the mutant DNMT3B protein has no ability to perform its canonical *de novo* methyltransferase function. It is possible a compensatory mechanism in the overexpressed cell lines may involve the induction of DNMT3A, the other DNMT3 family member.

Differentially methylated regions

In an effort to determine the statistical significance of the loss of DNA methylation in particular regions, all Gene Body, 3' UTR, Shore, and Shelf probes with a β -value of ≥ 0.75 in HCT116 were selected. The 0.75-1.0 range appeared to correspond with the most demethylation in the kernel density plots (Figure 2.3 D-E, G-H). Probes in the same position in 3BKO cells were compared while MT1KO and DKO served as controls. When DNMT3B was removed methylation changes were subtle, yet significant according to two-tailed paired t-tests (Figure 2.4). These changes are best evidenced by the difference in minimum β values. For example, in parental HCT116 the minimum Gene Body β -value is 0.75. In the 3BKO cells, the minimum Gene Body β -value is 0.02. Similarly, the minimum 3' UTR β -value in HCT116 is 0.75 compared to 0.09 in 3BKO. This trend holds true for shore and shelf methylation.

To thoroughly capture the effect of DNMT3B loss on DNA methylation, we examined DNA methylation decreases at all genomic signatures that were hypermethylated in HCT116 (β values ≥ 0.5) and hypomethylated in 3BKO (β values

≤ 0.2). A total of 314,096 probe positions were hypermethylated in the parental line (~6%). At the same position in 3BKO cells, 3,022 probes lost methylation (~1%) (Figure 2.5 A). Further supporting the notion this demethylation is under the control of DNMT3B, β values in 3BKO were very similar to those in DKO cells. Each signature had a slight gain in methylation in the 3BKO+WT3B samples not seen in the 3BKO+MUT3B cells (Figure 2.5 B). These 3,022 demethylated probes corresponded with 768 genes.

Comparison of CpG Island and non-CpG Island DNA methylation

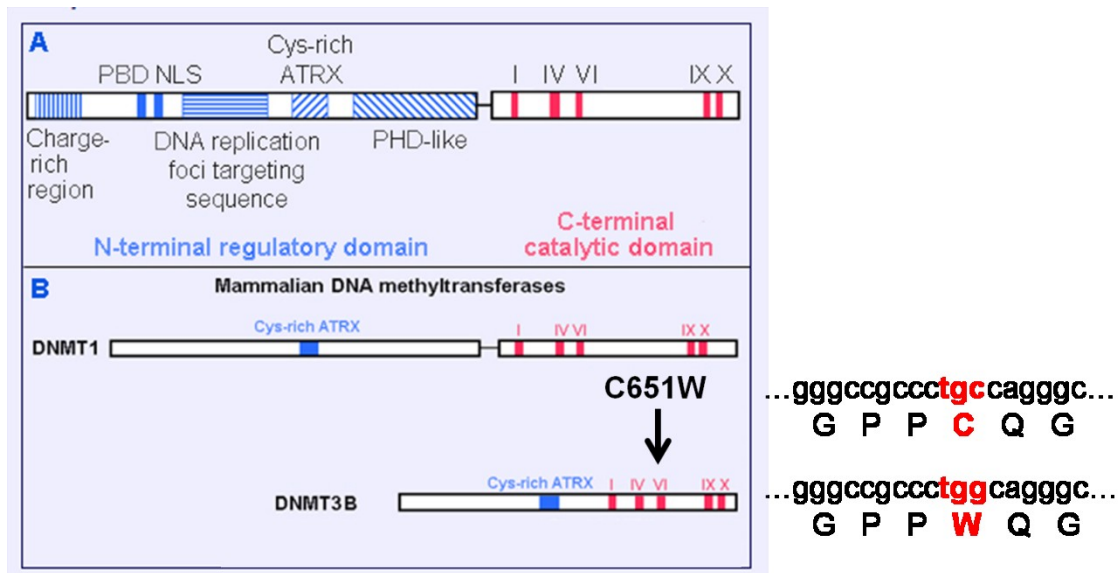
Since it is known that DNA methylation patterns can vary significantly between CpGs found inside and outside of a CpG island, and these shifts are associated with transcriptional changes, methylation levels between the promoter and gene body regions were compared. Much is known about the effect of methylation on promoters. Less is known about methylation changes in the gene body, and this genomic region was where we saw the greatest methylation changes. Interestingly, in our study the larger proportion of methylation changes were found in the non-CpG island genes in both promoter and gene body regions (Figure 2.6). The loss of DNMT3B seems to have a greater effect on the methylation status of non-CpG Island genes compared to CpG Island genes.

Relationship between DNA methylation and gene expression

To correlate DNA methylation changes with transcriptional changes, we compared the methylation patterns detected with our available gene expression data. Probes with the greatest degree of demethylation in both promoter and gene body regions were selected from both CpG categories (CpG island and non-CpG island). A bimodal distribution in expression was seen in both genomic regions, independent of CpG status.

This suggests the loss of DNMT3B-mediated DNA methylation alone is insufficient in regulating these genes.

A.



Adapted from: Arai, E. and Kanai Y., Atlas Genetics Cytogenetics Oncology Hematology 2009;13(1):p5-10. <http://atlasgeneticsoncology.org/Genes/DNMT1ID40347ch19p13.html>

B.

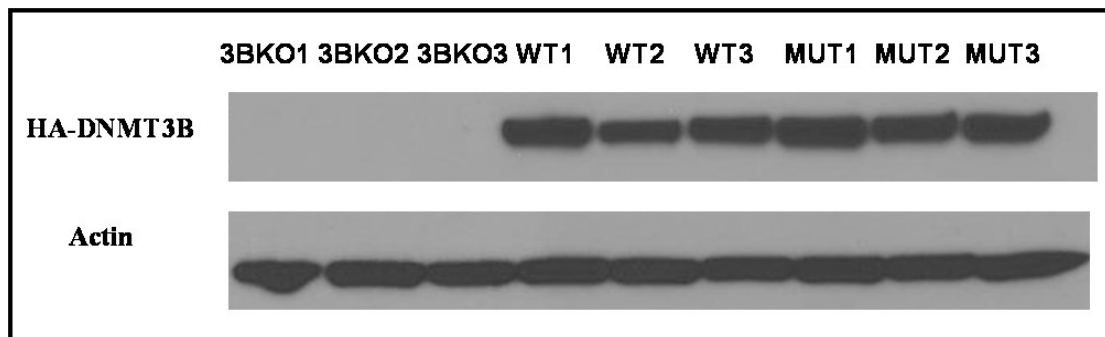
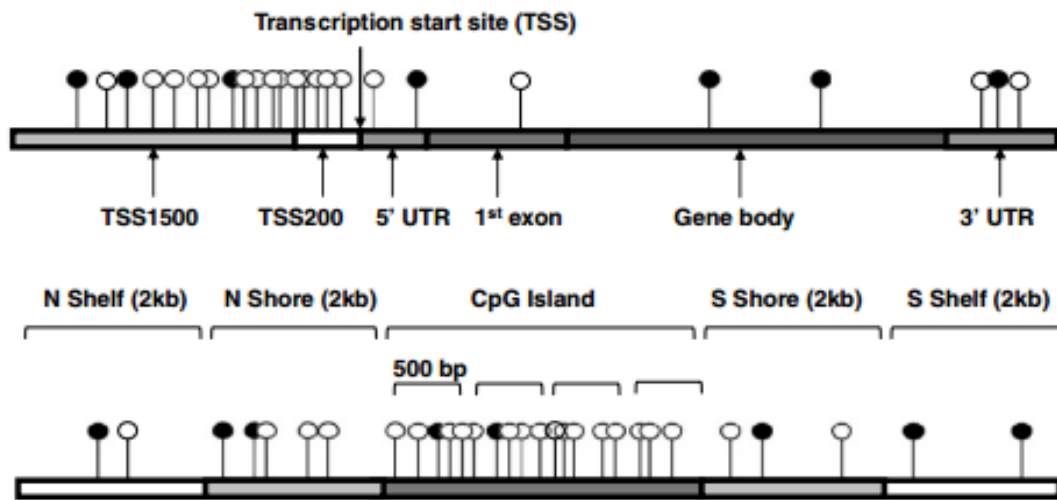


Figure 2.1. Transient transfection of wild-type (WT) and mutant (MUT) DNMT3B into *DNMT3B*^{-/-} cells. (A). Mutant DNMT3B (MUT) contains a point mutation in the catalytic domain (C651W) which abolishes all DNA methyltransferase activity. (B). A *DNMT3B*^{-/-} cell line was transiently transfected with empty vector alone (3BKO1-3), wild type HA-DNMT3B (WT1-3), and mutant HA-DNMT3B (MUT1-3) plasmid constructs.



Bibikova, M., *High density DNA methylation array with single CpG site resolution*. Genomics, 2011. 98(4): p. 288-295.

Figure 2.2. The Infinium HumanMethylation450 BeadChip Array Platform. Over 450,000 CpG probes are annotated at discrete functional regions of the human genome. The first two signatures are mapped relative to the Transcription Start Site (TSS). TSS1500 covers everything from -200 to -1500. TSS200 covers the 200 base pair region upstream of the TSS (+1). The 5' UTR, 1st exon, Gene Body and 3' UTR were also covered. CpG Islands and their associated Shore and Shelf probes were further distinguished.

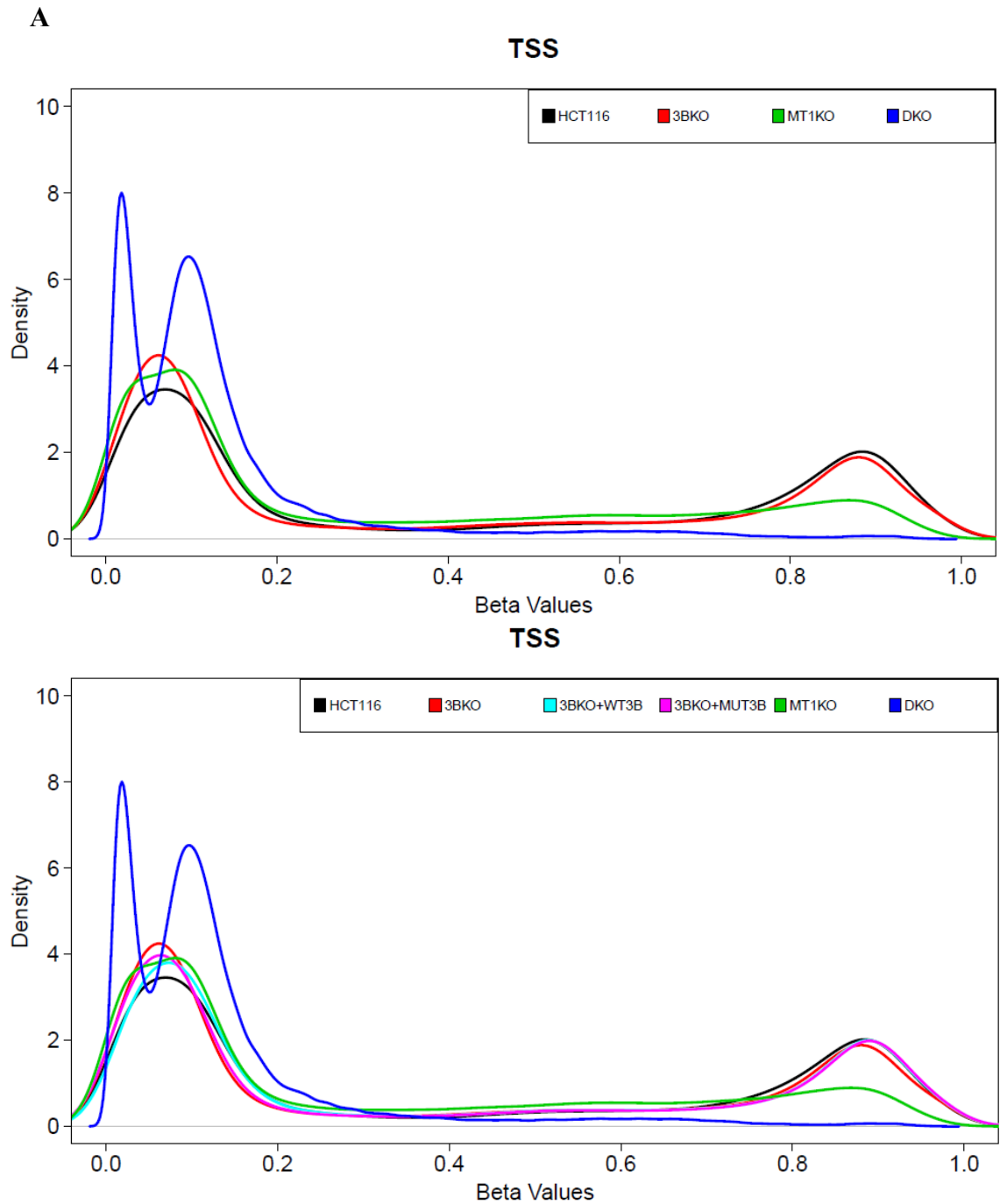


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (A) TSS1500 and TSS200 probes.

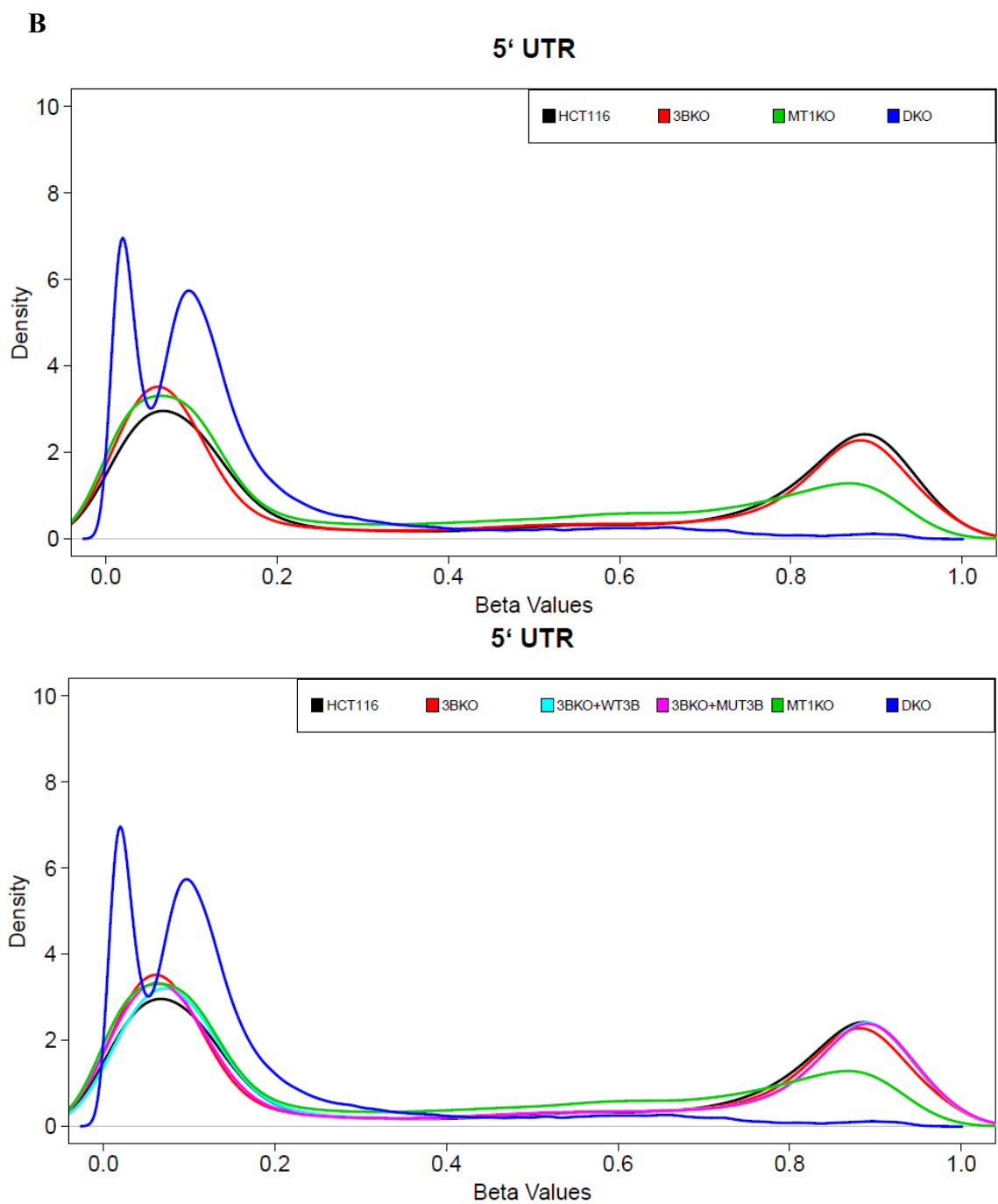


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (B) 5' UTR probes.

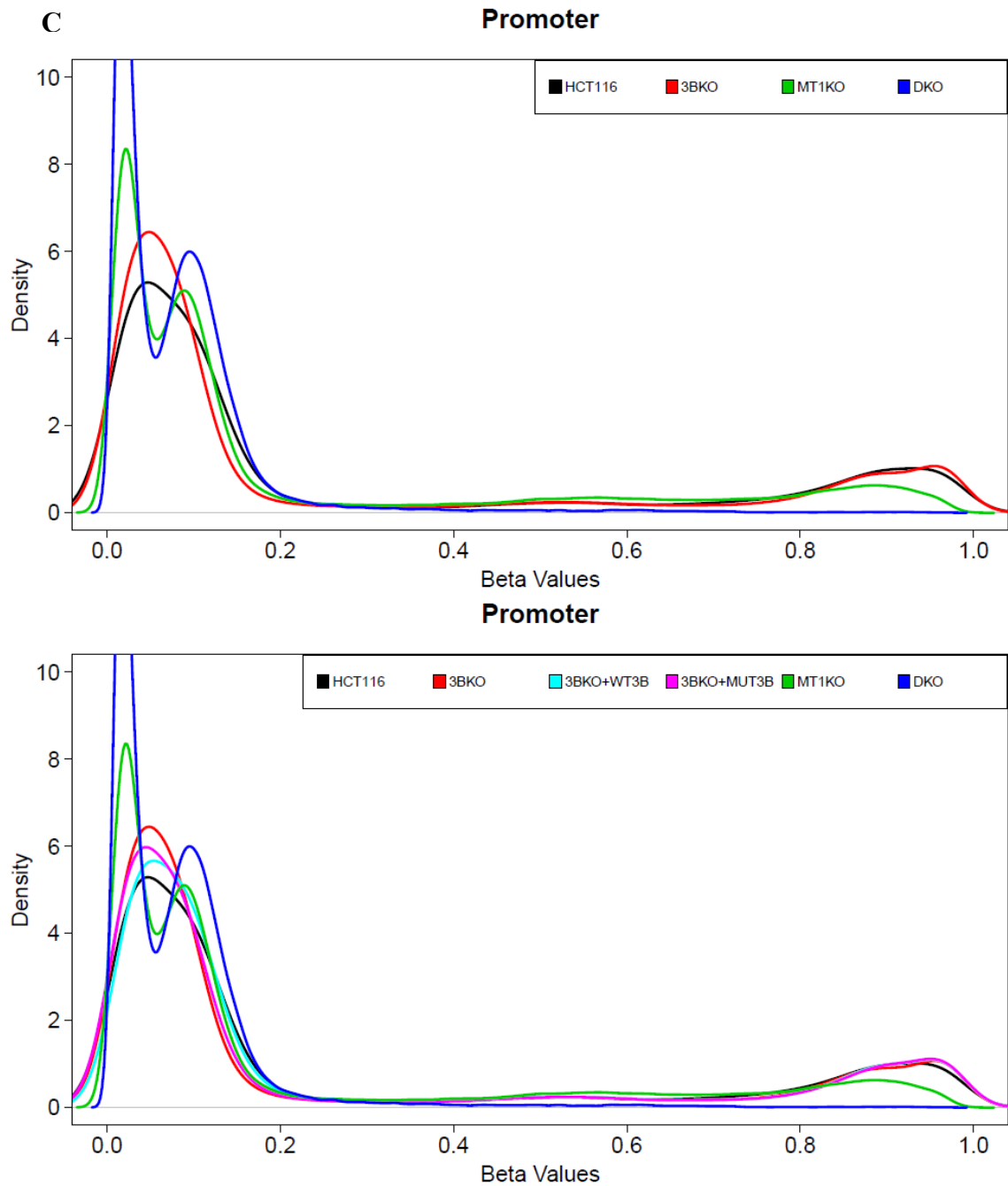


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (C) Promoter probes.

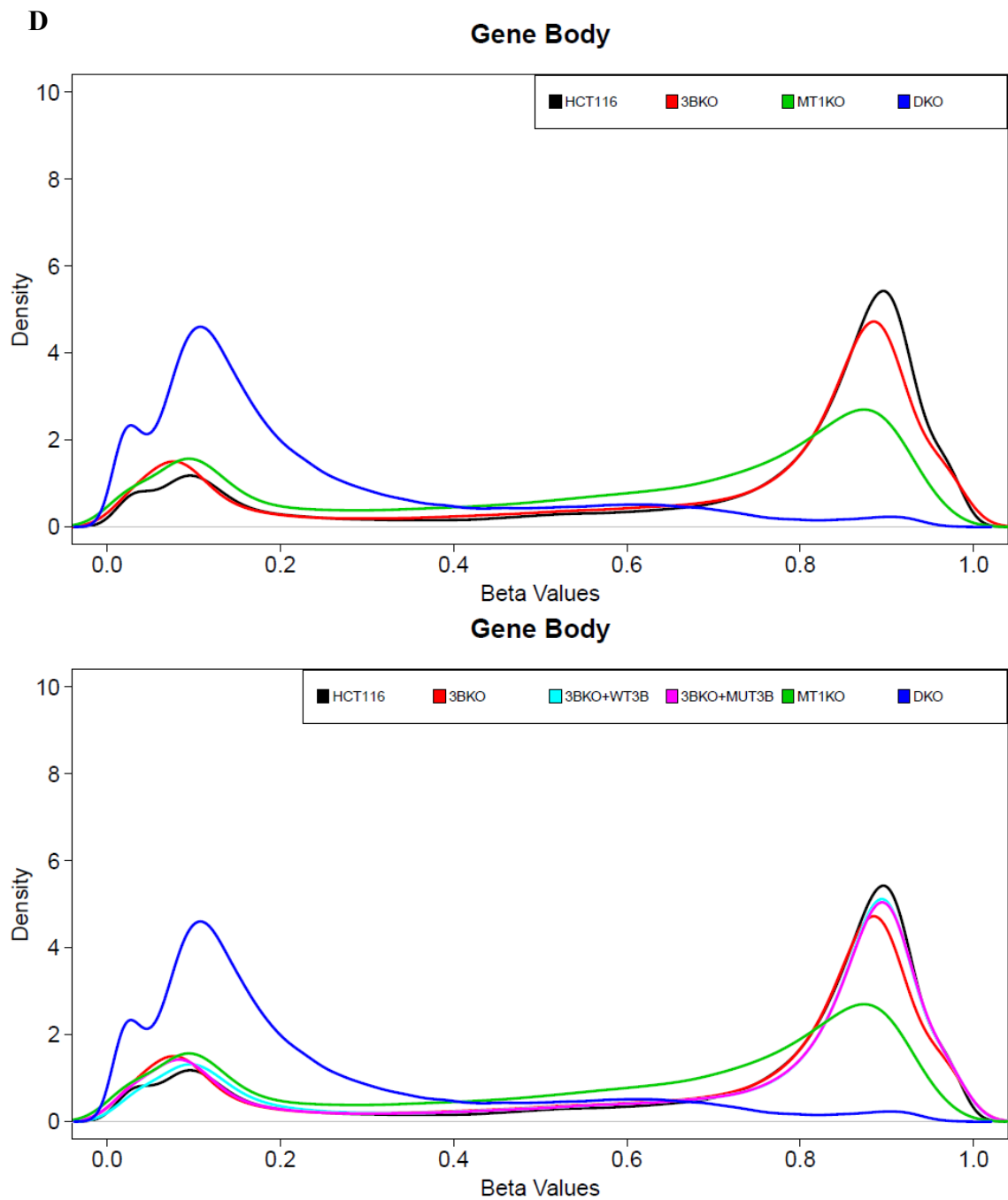


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (D) Gene Body probes.

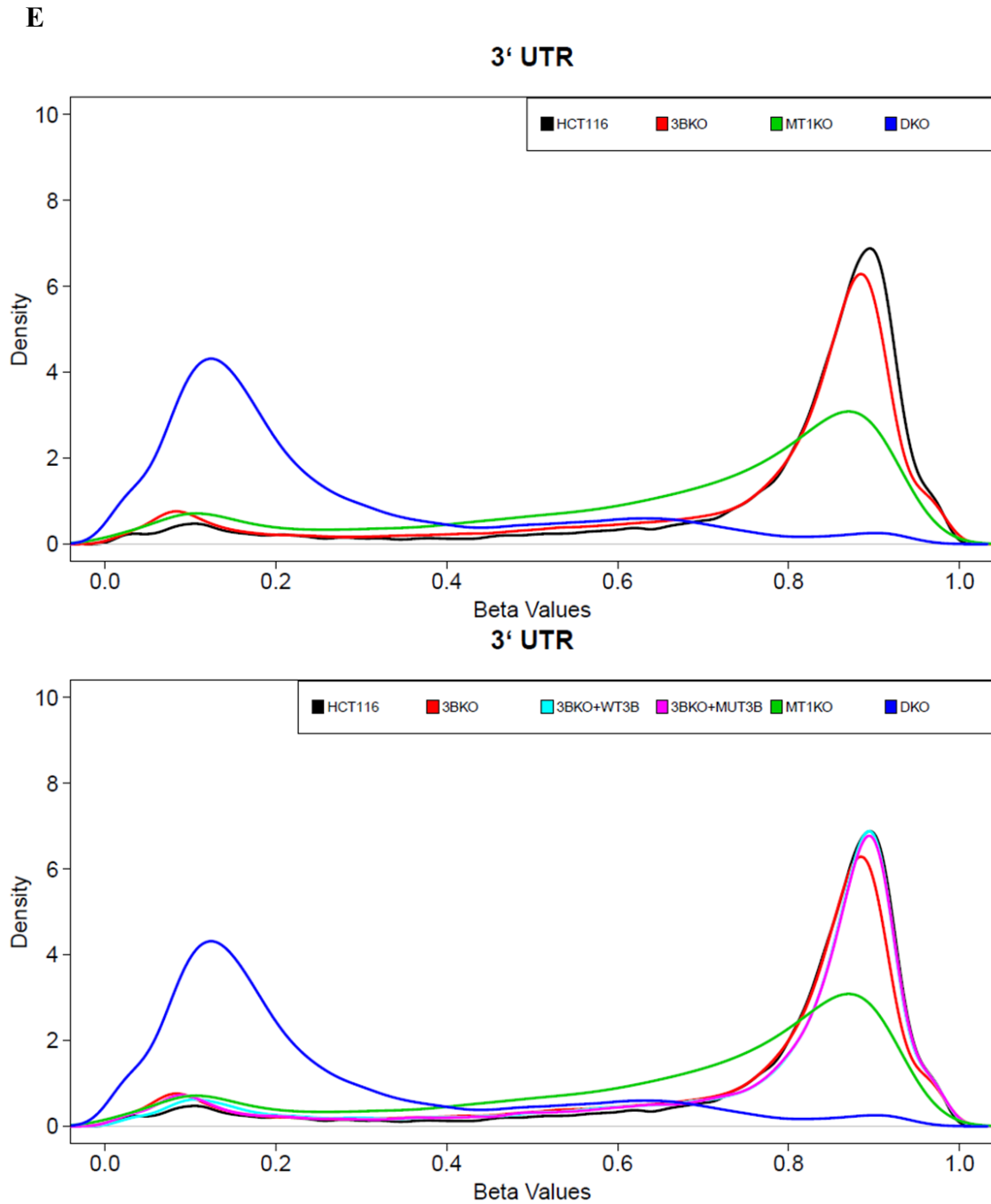


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (E) 3' UTR probes.

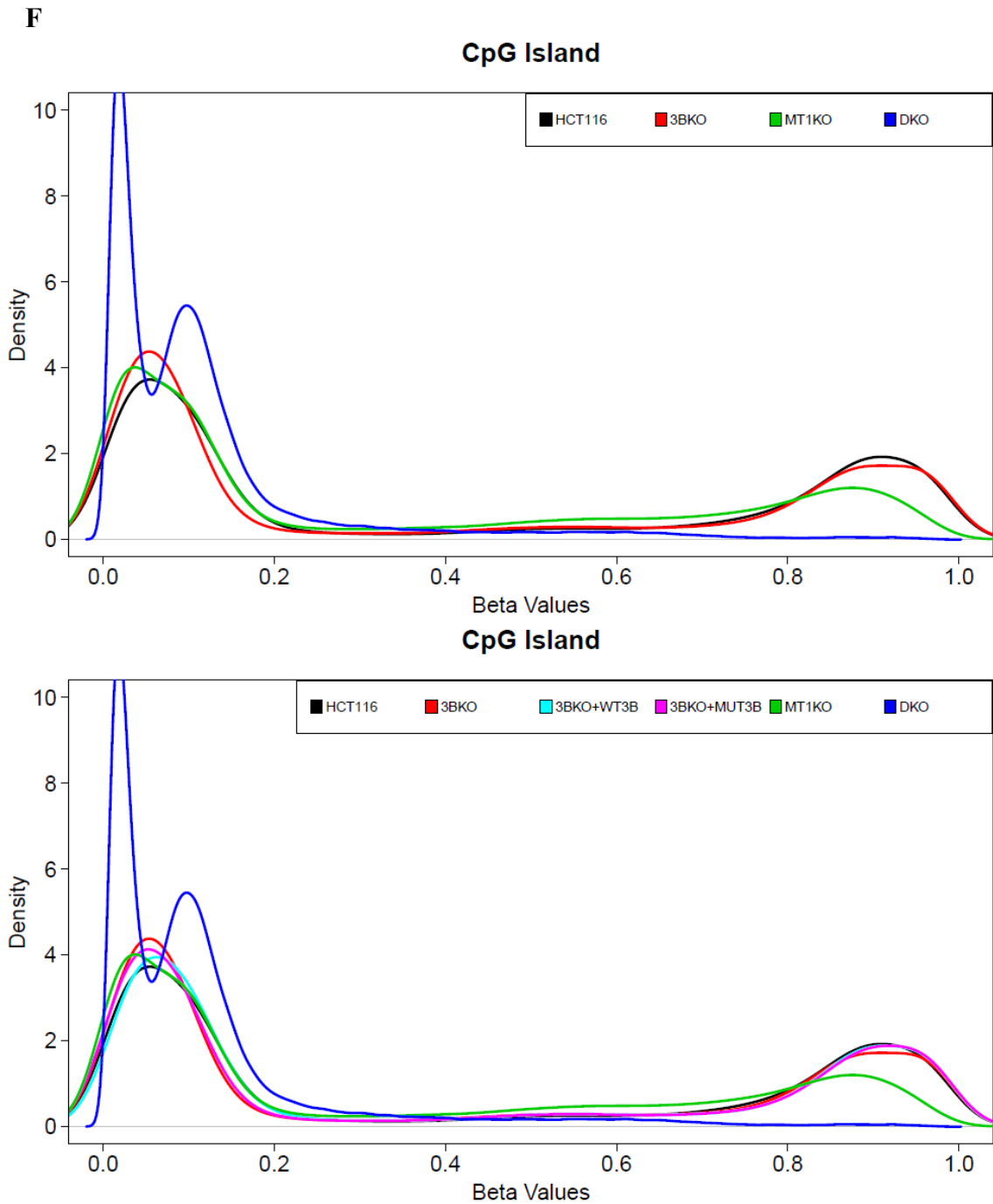


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (F) CpG Island probes.

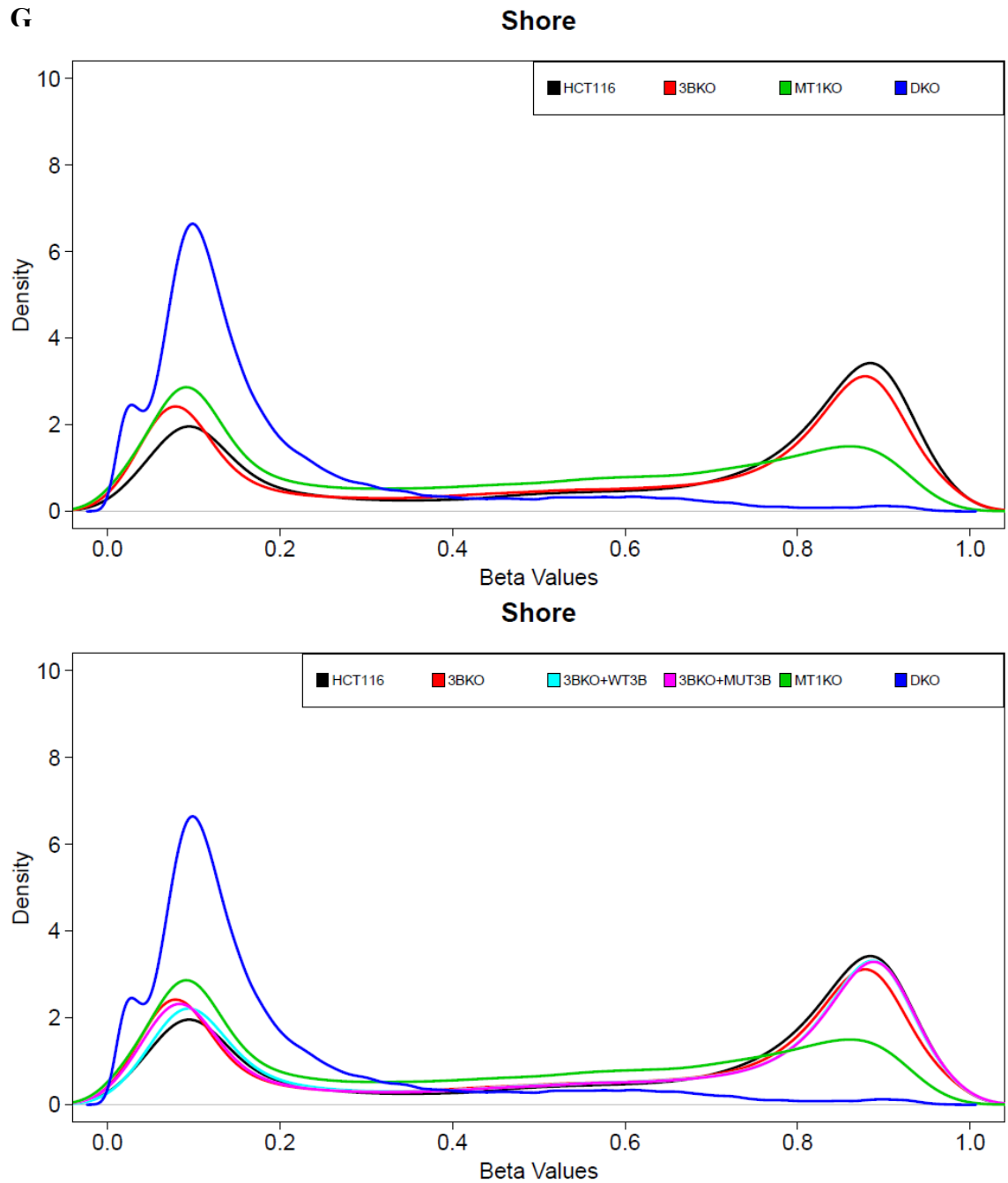


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (G) Shore probes.

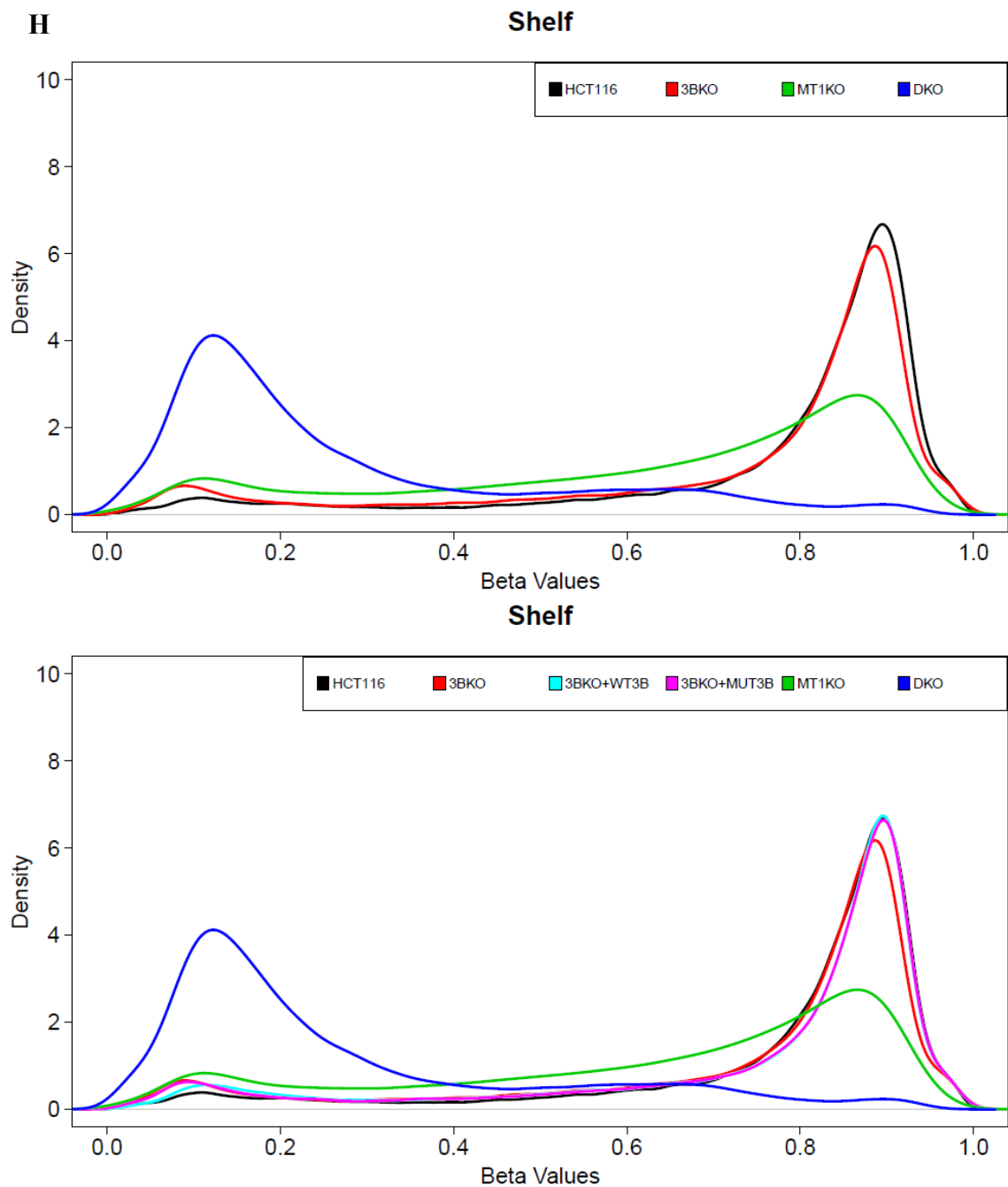


Figure 2.3. Global distribution of DNA methylation upon loss and overexpression of DNMT3B according to genomic location. β values closer to 0 = minimal methylation and β values closer to 1 = maximum methylation. (H) Shelf probes.

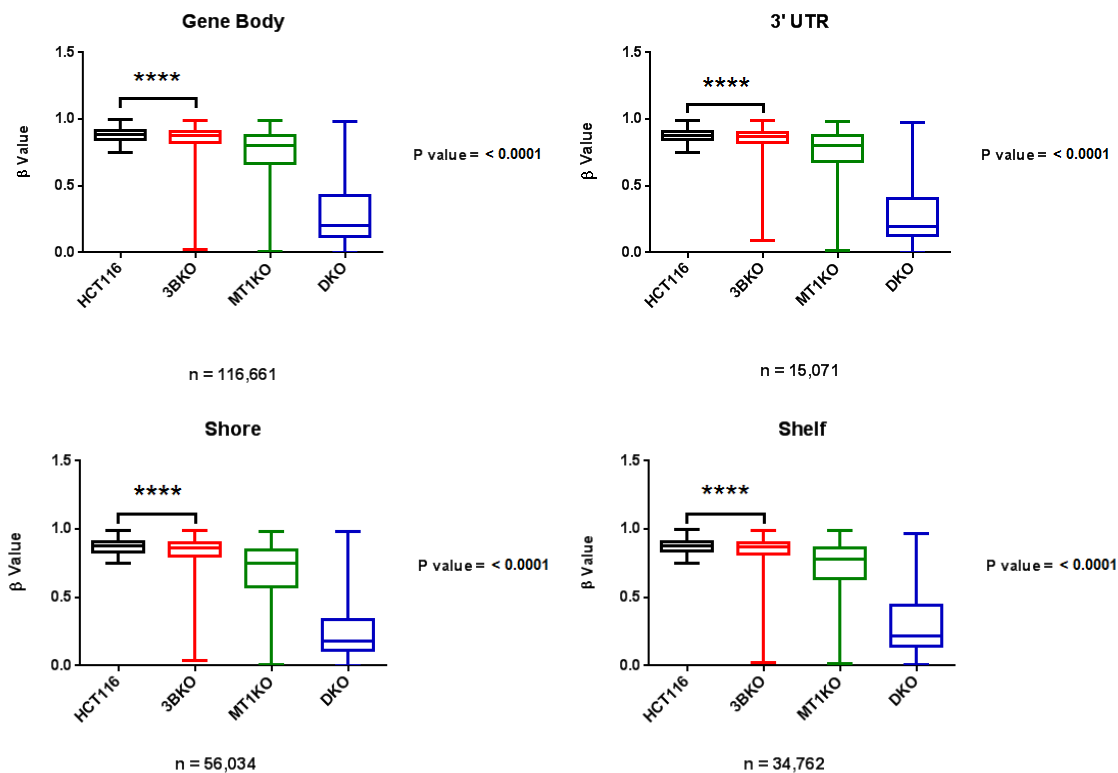


Figure 2.4. Paired comparison of hypermethylation in parental HCT116 relative to *DNMT3B*^{-/-} Cells. A two-tailed paired t-test was used to assess statistical significance of differentially methylated regions identified in kernel density plots depicting global methylation patterns.

A.

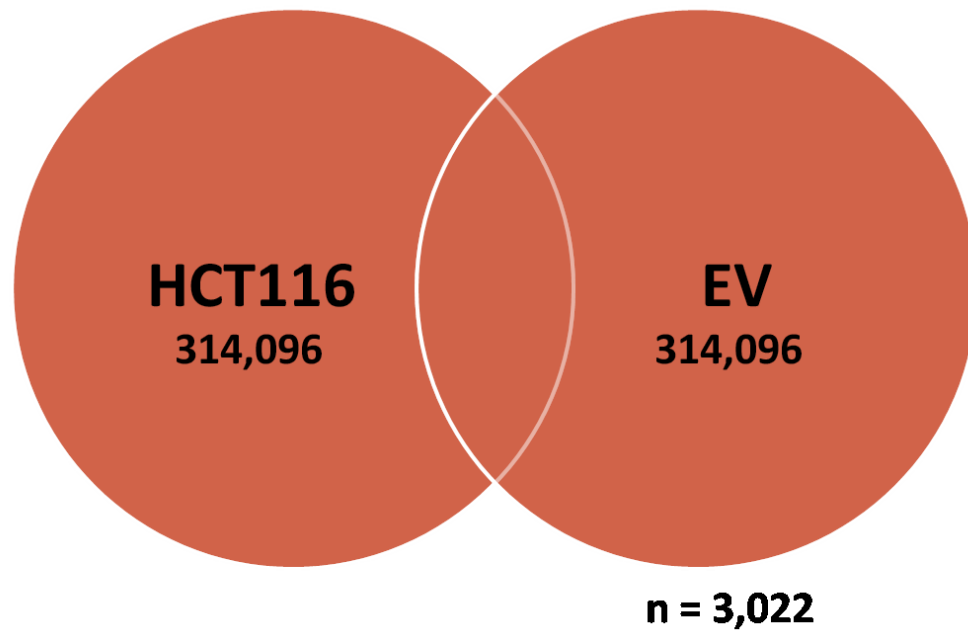


Figure 2.5. Paired Comparison of Hypermethylated Probes in Parental HCT116 (β values ≥ 0.50) to hypomethylated probes in *DNMT3B*^{-/-} Cells (β values ≤ 0.2). (A) Selected probe filtering. (B) Demethylated probe distribution according to genomic signature.

B.

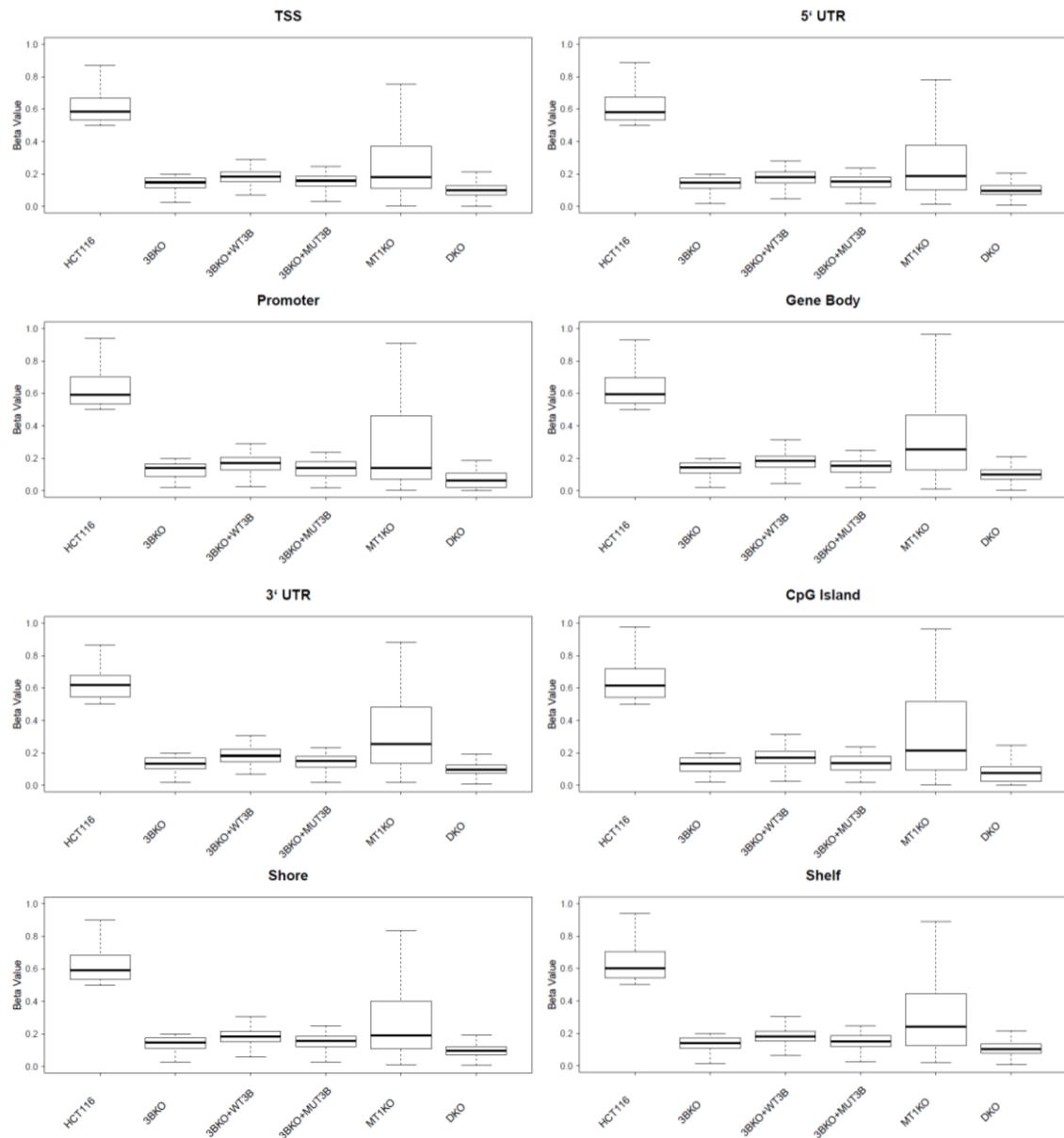


Figure 2.5. Paired Comparison of Hypermethylated Probes in Parental HCT116 (β values ≥ 0.50) to hypomethylated probes in *DNMT3B*^{-/-} Cells (β values ≤ 0.2). (A) Selected probe filtering. (B) Demethylated probe distribution according to genomic signature.

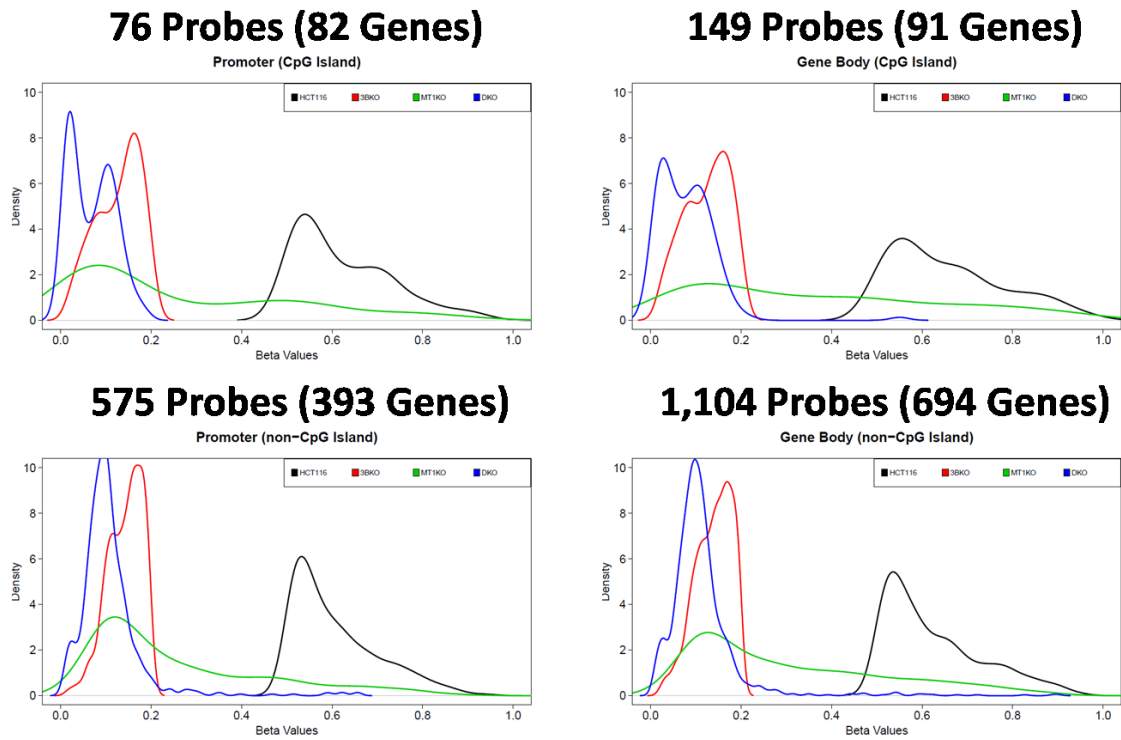


Figure 2.6. Identification of genes affected by loss of DNMT3B. Differentially demethylated probe distribution according to CpG island status of genomic signatures associated with transcriptional regulation. Top panels - CpG Island Genes; Bottom panels - non-CpG Island Genes. Black = HCT116, Red = 3BKO, Green = MT1 hypomorph, Blue = DKO).

A.

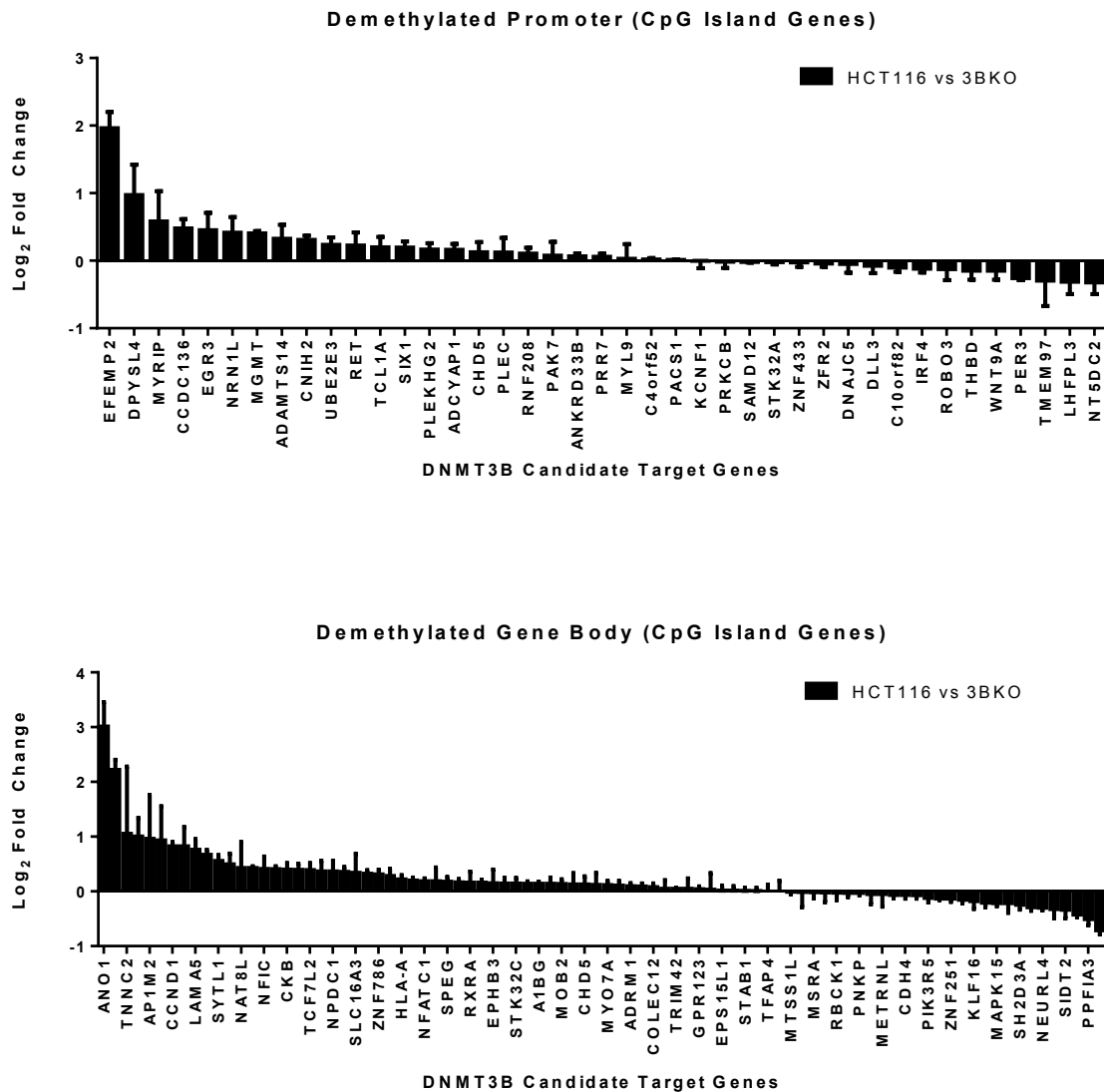


Figure 2.7. Expression status of genes that correspond with increased probe methylation. Genes that corresponded with the most demethylated probes were selected for analysis. (A) CpG island genes (B) non-CpG island genes

B.

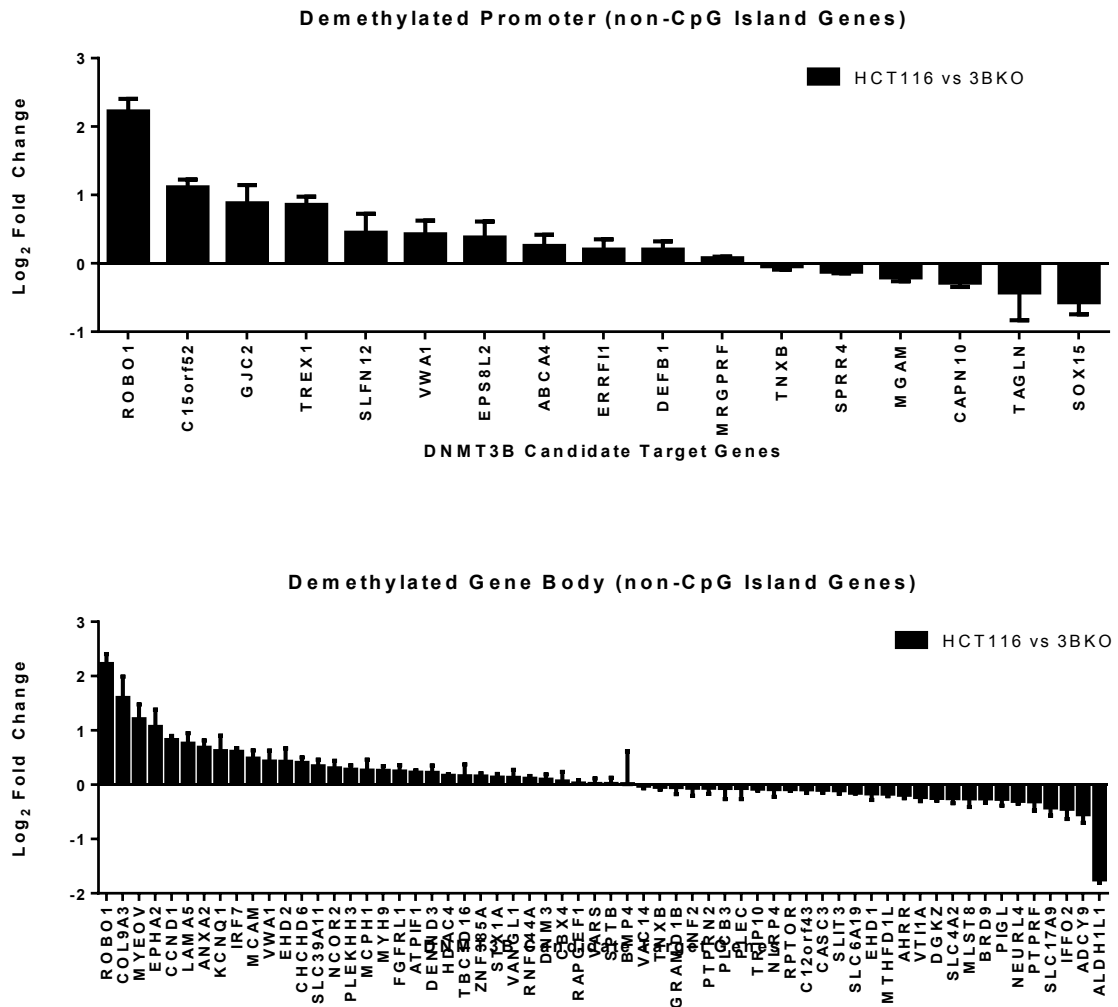


Figure 2.7. Expression status of genes that correspond with increased probe methylation. Genes that corresponded with the most demethylated probes were selected for analysis. (A) CpG island genes (B) non-CpG island genes

Discussion

Methylation has a complex relationship with gene expression. The most is known about DNA methylation dynamics at promoter regions. More than half of human genes contain a CpG island, which overlaps the TSS at 60-70% of human genes [6, 26]. When located at a TSS, most of these CpG islands are unmethylated.. In regards to CpG content, ~72% of promoters have high and 28% have low CpG content [26]. Low CpG density regions are frequently located outside of the TSS, suggesting 2 functional classes of promoters. When CpG islands are methylated around the TSS, this blocks the start of transcription and is associated with long-term gene silencing. DNA methylation dynamics in other genomic regions, and the correlation to gene expression, are currently under investigation.

Gene body methylation is one such area. This study reported a subtle loss of methylation across the genome upon DNMT3B removal, with the greatest changes occurring in Gene Body regions. CpG islands found in gene bodies can be methylated in a tissue-specific manner. By comparison, non-CpG island methylation is even more tissue-specific [6]. Gene body methylation can silence transposable elements but allow the host gene to continue transcriptional elongation. It is also known to contribute to cancer-causing somatic and germline mutations [6]. From our current understanding, the biological role of gene body methylation can vary. Evidence suggests it is a feature of transcribed genes. This notion is supported by X-chromosome work, and plant and animal shotgun bisulfite sequencing studies [6]. When H3K9me3 and MECP2 are found near methylation around the TSS, they repress transcription. However, these epigenetic players do not seem to have the same role at gene bodies. In this setting, transcription is not repressed. In fact, it seems gene body methylation promotes transcription elongation,

and thus gene activity. Although this study focused on cancer, gene body methylation seems to govern normal development as well. Up to 34% intragenic CpG islands are methylated in normal brains while the TSS remain largely unmethylated, suggesting transcriptional activity. The biological role of such a high degree of methylation remains unclear.

Gain of methylation lost in a transient DNMT3B overexpression setting was unexpected. It is possible DNMT3B may not have been acting alone. Dnmt3a and Dnmt3b directly interact via DNMT3L, stimulate each other's activity, and work synergistically at target gene promoters [27, 28]. DNMT3L complexes with both DNMT3A and DNMT3B to participate in *de novo* DNA methylation. When DNMT3L complexes with particular transcription factors, that can guide a DNMT3L/DNMT3A/transcription factor complex to target DNA sequences for methylation [29]. Further exploration is needed to determine if DNMT3B has the same mechanism in our system.

The paramount question is if any genes become silenced or expressed by the loss of DNMT3B-dependent methylation. The current model of how DNA methylation relates to gene expression would have predicted upregulation of genes that lost methylation in the promoter region and downregulation of genes that lost methylation in the gene body. The genes that experienced the most CpG island demethylation in the gene body regions also correlated with the most upregulation. This exception was also recently reported by another lab in cancer cell lines [30]. Perhaps, there is a subset of CpG island genes in the gene body that have abundant methylation that correspond with gene repression. This raises new questions about functional subclasses of gene body methylation.

Acknowledgements

Dr. Joo Mi Yi assisted with cloning and helpful discussions about cloning strategies. Dr. Eriko G. Clements provided useful discussions in regards to project ideas and experimental design. Dr. Hariharan Easwaran and Muhammad Noon helped with the DNA methylation and gene expression microarray analyses. Dr. Heather O'Hagan and Dr. Robert Casero contributed helpful and constructive feedback in the preparation of this manuscript.

References

1. Bird, A.P., *CpG-rich islands and the function of DNA methylation*. Nature, 1986. 321(6067): p. 209-13.
2. Heyn, H. and M. Esteller, *DNA methylation profiling in the clinic: applications and challenges*. Nat Rev Genet, 2012. 13(10): p. 679-92.
3. Baylin, S.B., et al., *Alterations in DNA methylation: a fundamental aspect of neoplasia*. Adv Cancer Res, 1998. 72: p. 141-96.
4. Law, J.A. and S.E. Jacobsen, *Establishing, maintaining and modifying DNA methylation patterns in plants and animals*. Nat Rev Genet, 2010. 11(3): p. 204-20.
5. Zemach, A., et al., *Genome-wide evolutionary analysis of eukaryotic DNA methylation*. Science, 2010. 328(5980): p. 916-9.
6. Jones, P.A., *Functions of DNA methylation: islands, start sites, gene bodies and beyond*. Nat Rev Genet, 2012. 13(7): p. 484-92.
7. Feng, S., et al., *Conservation and divergence of methylation patterning in plants and animals*. Proc Natl Acad Sci U S A, 2010. 107(19): p. 8689-94.
8. Wu, H., et al., *Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes*. Science, 2010. 329(5990): p. 444-8.
9. Foret, S., et al., *DNA methylation dynamics, metabolic fluxes, gene splicing, and alternative phenotypes in honey bees*. Proc Natl Acad Sci U S A, 2012. 109(13): p. 4968-73.
10. *Insights into social insects from the genome of the honeybee Apis mellifera*. Nature, 2006. 443(7114): p. 931-49.

11. Kucharski, R., et al., *Nutritional control of reproductive status in honeybees via DNA methylation*. Science, 2008. 319(5871): p. 1827-30.
12. Goll, M.G. and T.H. Bestor, *Eukaryotic cytosine methyltransferases*. Annu Rev Biochem, 2005. 74: p. 481-514.
13. Aran, D., et al., *Replication timing-related and gene body-specific methylation of active human genes*. Hum Mol Genet, 2011. 20(4): p. 670-80.
14. Jin, B., et al., *DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function*. Hum Mol Genet, 2008. 17(5): p. 690-709.
15. Hansen, R.S., et al., *The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome*. Proc Natl Acad Sci U S A, 1999. 96(25): p. 14412-7.
16. Bachman, K.E., M.R. Rountree, and S.B. Baylin, *Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin*. J Biol Chem, 2001. 276(34): p. 32282-7.
17. Velasco, G., et al., *Dnmt3b recruitment through E2F6 transcriptional repressor mediates germ-line gene silencing in murine somatic tissues*. Proc Natl Acad Sci U S A, 2010. 107(20): p. 9281-6.
18. Myant, K. and I. Stancheva, *LSH cooperates with DNA methyltransferases to repress transcription*. Mol Cell Biol, 2008. 28(1): p. 215-26.

19. Linhart, H.G., et al., *Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing*. Genes Dev, 2007. 21(23): p. 3110-22.
20. Kim, S.H., et al., *Zinc-fingers and homeoboxes 1 (ZHX1) binds DNA methyltransferase (DNMT) 3B to enhance DNMT3B-mediated transcriptional repression*. Biochem Biophys Res Commun, 2007. 355(2): p. 318-23.
21. Kleinman, C.L., et al., *Fusion of TTYH1 with the C19MC microRNA cluster drives expression of a brain-specific DNMT3B isoform in the embryonal brain tumor ETMR*. Nat Genet, 2014. 46(1): p. 39-44.
22. Rhee, I., et al., *DNMT1 and DNMT3b cooperate to silence genes in human cancer cells*. Nature, 2002. 416(6880): p. 552-6.
23. Rhee, I., et al., *CpG methylation is maintained in human cancer cells lacking DNMT1*. Nature, 2000. 404(6781): p. 1003-7.
24. Bibikova, M., et al., *High density DNA methylation array with single CpG site resolution*. Genomics, 2011. 98(4): p. 288-95.
25. Bestor, T.H., *The DNA methyltransferases of mammals*. Hum Mol Genet, 2000. 9(16): p. 2395-402.
26. Saxonov, S., P. Berg, and D.L. Brutlag, *A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters*. Proc Natl Acad Sci U S A, 2006. 103(5): p. 1412-7.
27. Li, J.Y., et al., *Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog*. Mol Cell Biol, 2007. 27(24): p. 8748-59.

28. Van Emburgh, B.O. and K.D. Robertson, *Modulation of Dnmt3b function in vitro by interactions with Dnmt3L, Dnmt3a and Dnmt3b splice variants*. Nucleic Acids Res, 2011. 39(12): p. 4984-5002.
29. Pacaud, R., et al., *DNMT3L interacts with transcription factors to target DNMT3L/DNMT3B to specific DNA sequences: role of the DNMT3L/DNMT3B/p65-NFkappaB complex in the (de-)methylation of TRAF1*. Biochimie, 2014.
30. Varley, K.E., et al., *Dynamic DNA methylation across diverse human cell lines and tissues*. Genome Res, 2013. 23(3): p. 555-67.

Chapter 3

DNMT3B (a *de novo* DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity

Abstract

Human cancers are characterized by aberrant DNA methylation patterns, including global hypomethylation and hypermethylation at promoters of tumor suppressor genes, leading to transcriptional silencing. The *de novo* methyltransferases, DNMT3A and DNMT3B, establish new methylation patterns. DNMT1, the maintenance methyltransferase, ensures propagation of hemi-methylated. Experimentally, DNMT1 is capable of transcriptional repression without its methyltransferase activity, partially through interactions with histone-modifying enzymes. We hypothesized DNMT3B may also be capable of modulating gene expression independently of its methyltransferase activity, by recruiting repressive epigenetic proteins to the promoters of endogenous DNMT3B targets.

Using an isogenic colorectal cancer cell culture model, we investigated the functional consequences of the removal and re-introduction of DNMT3B on target gene expression, methylation, and chromatin status. A stable genetic knock out cell line and pharmacologic inhibition resulted in upregulation of candidate DNMT3B targets. Interestingly, reintroduction of both wild-type and catalytically dead mutant DNMT3B in 3BKO cells transcriptionally repressed the same loci. Infinium 450K arrays reported a loss of DNMT3B did not result in significant changes in global methylation at TSS, 5' UTR, Promoter (-500 --> +1500), CpG Island, and Shore regions. However, significant changes were identified in Gene Body, 3' UTR, and Shelf signatures. Overexpression of both wild-type and catalytically dead DNMT3B failed to restore DNA methylation.

Interestingly, DNMT3B removal had a greater effect on DNA methylation in the non-CpG island gene signatures. The lack of basal DNA methylation at the promoters of

CpG island genes, in concert with transcriptional repression of these loci, further suggest an alternative regulatory mechanism to explain transcriptional changes. DNMT3B was recruited back to endogenous target genes and LSD1 recruitment mirrored DNMT3B recruitment. Loss of DNMT3B correlated with an increase in an active histone mark while a repressive histone mark remained unchanged. Together, this study suggests DNMT3B can regulate gene expression in two independent fashions. DNMT3B can methylate DNA in a region-specific manner throughout the genome, suggesting C-terminal domain activity. However, without its catalytic activity, DNMT3B may mediate gene repression by partial interactions with histone modifying enzymes on its N-terminal end.

Introduction

DNA methylation is essential for controlling normal biological processes like mammalian development, X-chromosome inactivation, genomic imprinting, chromatin structure, and gene expression. Human cancers are characterized by aberrant DNA methylation patterns, such as global hypomethylation and hypermethylation at promoters of tumor suppressor genes, leading to transcriptional silencing. Gene expression changes can be regulated by enzymes that chemically modify genomic DNA or histones, as well as chromatin remodeling factors that regulate chromatin accessibility.

DNA methyltransferases are a family of enzymes that catalyze the transfer of methyl groups to DNA. DNMT1, the maintenance methyltransferase, has a preference for hemi-methylated DNA and ensures the faithful propagation of DNA methylation patterns on newly synthesized DNA. The *de novo* methyltransferases, DNMT3A and DNMT3B, establish new methylation patterns during early development. DNMT1 is localized to DNA replication foci by UHRF1. DNMT3A and DNMT3B lack a targeting co-repressor and no DNMT recognition site or sequence has been reported to date.

All DNMTs share a highly conserved C-terminal catalytic domain, with distinctive N-terminal domains. The N-terminal domain is thought to direct nuclear localization and interact with other proteins to mediate transcriptional activity. DNMT3B, unlike DNMT1 and 3a, is the only DNMT3 with alternatively spliced variants affecting the catalytic domain [1]. The specific roles of these variants in cancer are not understood but it suggests the catalytic domain may be dispensable for transcriptional regulation.

Experimentally, DNMT1 is capable of transcriptional repression without its methyltransferase activity, partially through interactions with histone-modifying enzymes

[2]. The study identified endogenous DNMT1 target genes and demonstrated the protein represses target genes independent of its catalytic activity. DNA methylation was not required for gene repression, even though DNMT1 was recruited back to target promoters [2]. When chromatin status was assessed active marks were depleted in the presence of both wild type and mutant DNMT1, but repressive marks were unchanged with loss of DNMT1 alone [2].

DNMT3B having functions other than the ability to methylate DNA is not unheard of. Dnmt3b represses transcription in a methylation-independent manner [3]. This may be through the N-terminal domain of DNMT3B which is known to associate with HDAC2 [4]. Tdg and Mbd4 make multiple interactions with Dnmt3b *in vivo*, with reduced T·G mismatch repair efficiency upon loss of DNMT3B [5]. DNMT1, DNMT3B, PRC4 components complex together and are targeted to CpG island promoters upon oxidative damage [6]. DNMT3B has also been reported to cooperate with DNMT1 to maintain methylation in the human genome [7], which is a deviation from its role in early development. Thus, it is possible DNMT1 and DNMT3B may operate using similar regulatory mechanisms. We hypothesized DNMT3B may also be capable of modulating gene expression independently of its methyltransferase activity, by recruiting repressive epigenetic proteins to the promoters of endogenous DNMT3B targets.

Materials and Methods

Cell culture

HCT116, *DNMT1*^{-/-} hypomorph subclone 5F (MT1KO), *DNMT3B*^{-/-} subclone 2 (3BKO), and *DNMT1*^{-/-} hypomorph; *DNMT3B*^{-/-} subclone 2 (DKO) were previously described [7, 8]. All cell lines were cultured in McCoy's 5A (Iwakata & Grace Modification) (Cat No. 10-050-CV, Corning cellgro) supplemented with 10% fetal bovine serum (Cat No. 35-011-CV, Corning cellgro) at 37 °C and 5% CO₂ atmosphere.

RNA isolation

RNeasy Mini Kit™ (Cat No. 74104, Qiagen) was used to extract 1 µg total RNA from each cell line according to manufacturer's protocol. Isolated total RNA was treated with RNase-Free DNase I (Cat No. 79254, Qiagen).

Gene expression analysis with human gene expression microarrays

Total RNA was hybridized to Human GE 4x44K v2 Microarray (Cat. No. G4845A, Agilent) according to manufacturer's protocol.

Expression data processing and visualization

The Bioconductor and Limma packages in the R programming and software environment were used to further analyze and visualize expression data. Log₂ ratios were calculated. Genes with a Log₂ ratio of ≥ 0.5 were described as upregulated and ≤ -0.5 were considered downregulated.

cDNA synthesis

One µg total RNA was reversed transcribed using SuperScript III First Strand cDNA Synthesis Kit and oligo(dT20) primers (Cat. No. 18080-051, Invitrogen). For every 20

μL prep, 80 μL dd water was added. Five μL of diluted cDNA was used per qPCR reaction. Minus RT and water control reactions preps were included.

Quantitative PCR

The QuantiTect SYBR Green PCR Kit was used to perform all PCRs (Cat. No. 204243, Qiagen). (See Table 3.1 for primers)

DNMT Inhibitor Treatment

Parental HCT116 and 3BKO cell lines were both treated with 500 nM 2'-deoxy-5-azacytidine (DAC) for 72 hours. PBS was used as a mock control.

Plasmid constructs

Wild type *DNMT3B* Isoform 1 (NM_006892.3) with N-terminal HA tag inserts were subcloned into a pEF1α IRES-puro vector (WT3B). A QuikChange II Site-Directed Mutagenesis Kit (Cat No. 200523, Agilent) was used to generate a catalytically inactive *DNMT3B* (C651W) (MUT3B).

Transient transfection

The *DNMT3B*^{-/-} subclone 2 (3BKO) cell line was transiently transfected for 48 hours with 1 μg of both wild type and mutant plasmid DNA using Lipofectamine 2000 (Cat No. 11668-027, Life Technologies).

Genomic DNA isolation

DNeasy Blood and Tissue Kit™ (Cat No. 69504, Qiagen) was used to extract 1 μg genomic DNA from each cell line according to manufacturer's protocol. Isolated genomic DNA was bisulfite converted using EZ-DNA Methylation™ Kit (Cat No. D5001, Zymo Research).

Methylation analysis with the Infinium HumanMethylation450 BeadChip Kit

DNA methylation analyses of the 9 experimental samples (biological triplicates of 3BKO, 3BKO overexpressing WT3B, and overexpressing MUT3B) and 3 control cell lines (HCT116, MT1KO, and DKO) were performed with the Infinium HumanMethylation450 BeadChip Kit (Cat. No. WG-314-1001, Illumina) according to the manufacturer's protocols. The platform contains 486,428 CpG sites distributed across the promoter (-500 --> +1500), 5' UTR, first exon, gene body, 3' UTR, CpG island, shore, and shelf regions and covers 99% of RefSeq genes [9].

Methylation data processing and visualization

Illumina GenomeStudio Methylation Module software (Part No. 11319130 Rev. B, Illumina) was used to calculate methylation levels and identify methylation signatures across the entire genome. Reported β -values reflect the estimated methylation level of each CpG locus using the ratio of intensities between methylated and unmethylated probes. The Bioconductor package in the R programming and software environment was used to further analyze and visualize methylation data.

Chromatin immunoprecipitation (ChIP)

Cells were crosslinked using 37 % formaldehyde. Nuclear extracts from $\sim 1 \times 10^6$ cells were used per IP. Crosslinked cells were resuspended in CEBN, followed by a CEB wash. Nuclear pellets were resuspended in SDS lysis buffer. Sonication was performed using the Bioruptor Pico (Cat No. B01060001, Diagenode). (Program: Time on=30 sec, Time off=30 sec, Cycle # =40. 60 μ g chromatin was used per IP. Antibodies (2-10 μ g): α -H3K4me3 (Cat No. 07-473, Millipore), α -H3K27me3 (Cat No. 07-449, Millipore), α -LSD1 (Cat No. ab17721, Abcam), α -DNMT3B (generated in-house)

Results

Identification of endogenous DNMT3B target genes.

Prior work in our lab has shown Dnmt3b lacking its methyltransferase domain can repress transcription of a reporter gene [3]. One drawback to these studies was the inability to investigate DNA methylation and chromatin modifications at endogenous promoters. We first identified DNMT3B targets using a genetic knockout of DNMT3B (3BKO) in HCT116 colorectal cancer cells [3]. In an effort to study the repressive effects of DNMT3B, genes that displayed increased expression in the 3BKO setting were identified through microarray analysis. In our initial filter, we identified 347 genes upregulated ≥ 0.5 fold in three different 3BKO clones compared to parental HCT116 (Figure 3.1 A). Next, we sought to determine how many of the 347 candidate genes were also upregulated in DKO cells, which lack DNMT3B and contain a DNMT1 hypomorph. Of the total 147 genes, 75 known DNMT1 targets were filtered out (Figure 3.1 B). The 72 remaining DNMT3B target genes were selected for further study. Approximately 2/3 of the genes were CpG island genes, as expected. Interestingly, $\sim 1/3$ were non-CpG island genes (Figure 3.1 B).

After analyzing microarray data for a subset of target genes (15/72) in various DNMT knockout settings, it was clear DNMT1 had a negligible effect on these genes. *MYC*, a gene that is transcriptionally active but not under the control of DNMT3B, served as a control (Figure 3.2 A). Quantitative RT-PCR validation showed all genes analyzed showed increased expression in 3BKO cells relative to parental HCT116 (Figure 3.2 B). To further validate that the genes were DNMT3B specific targets, a DNMT inhibitor was used in parental HCT116 and 3BKO cell lines (Figure 3.3). Increase in expression was

seen only in HCT116, not 3BKO cells. Thus, depletion of the other DNMTs via pharmacologic inhibition in the 3BKO had no effect on target gene expression.

DNMT3B methyltransferase activity is not required for gene repression

To address if the repressive effect of DNMT3B depends on the methyltransferase activity of the protein, we overexpressed wild type or catalytically inactive DNMT3B into 3BKO cells. The mutant constructs contains a point mutation at amino acid residue 651. A cysteine to tryptophan substitution abolishes all catalytic activity (previously described, see Figure 2.1) without altering the 3-dimensional structure of the DNMT3B [10]. An HA tag was added to the N-terminus portion of DNMT3B and the constructs were transiently transfected. Western blot analysis indicates robust expression of the two DNMT3B species to similar degrees, independent of DNMT3B mutation status (Figure 2.1). Interestingly, reintroduction of both wild type and catalytically dead DNMT3B in the knockout cells transcriptionally repressed most target genes (Figure 3.4). This result suggests putting back wild type or mutant DNMT3B both have similar expression profiles. Together, these data are consistent with our hypothesis that DNMT3B activity is not required for gene repression.

DNA methylation is not required for gene repression

Infinium 450K arrays were used to determine the effects of DNMT3B removal and reintroduction on methylation across the genome of the 72 DNMT3B target genes. Previous work reported the genetic removal of DNMT3B resulted in a ~3% decrease in global genomic methylation [7]. However, the region specificity of that loss could not be ascertained due to assay limitations at the time. Methylation arrays afforded the opportunity to detect global methylation patterns and region specific changes that can

help explain DNMT3B targeting. The methylation status of 8 genomic signatures (TSS, 5' UTR, Promoter, Gene Body, 3' UTR, CpG Island, Shore, and Shelf) were assessed (Figure 2.2).

Parental HCT116 was the most hypermethylated cell line used in this study and DKO had the least methylation. Single knockout mutants displayed varying degrees of methylation. Our focused analysis centered on 3BKO cells. Kernel density revealed changes in most regions were minimal (Figure 3.5 A-C, F-G). In TSS, 5' UTR, Promoter, CpG Island, and Shore regions methylation values failed to surpass a density of 3.0 in HCT116, reflecting little baseline methylation. Low basal methylation in the parental line explains the failure to detect appreciable loss of methylation at these signatures in the 3BKO cells.

Comparatively, Gene Body, 3' UTR, and Shelf regions were more densely methylated in HCT116 and displayed decreased methylation in the absence of DNMT3B (Figure 3.5 D-E, H). DNA methylation appears to be more prominent and readily lost in the areas where transcription is not initiated (Gene Body, 3' UTR, and Shelf regions). Overexpression of both the wild type and mutant DNMT3B failed to restore methylation in these regions (Figure 3.5 D-E, H). Gene bodies start with much more methylation than other genomic regions. Some effect is observed by removing DNMT3B alone. However, DNMT1 also has an effect on the methylation of this region. We can surmise that both enzymes may be involved with its regulation. This is further evidenced by the complete loss of methylation in the DKO cells. Of the 8 genomic regions queried, only 3 had noticeable DNA methylation changes in the 3BKO setting. In an effort to test the significance of these changes, statistical analyses were performed. Pairwise comparisons

were made between differentially methylation regions in parental HCT116 and 3BKO cells (Figure 3.6). Changes in methylation are most easily noted by the drop in minimum methylation values on the box-and-whisker plots. These regions started with a greater amount of basal DNA methylation in HCT116 and showed statistically significant decreases in methylation in 3BKO cells according to two-tailed paired t tests.

Global DNA methylation patterns according to CpG island status

Next, we sought to determine if DNA methylation patterns varied at genomic regions according to CpG island status. Hypermethylation of CpG islands in promoter regions is associated with gene repression, while hypomethylation corresponds with transcriptional activity. Although not definitive, the converse is thought to be true for CpG islands found in the gene body. A high level of gene body methylation is commonly thought to promote transcription.

To further elucidate DNMT3B's role in this "DNA methylation paradox", we subdivided our 72 DNMT3B target genes into two categories; CpG island (46) and non-CpG island (26) genes. Loss of DNMT3B has a greater effect on DNA methylation in the promoter regions of non-CpG island genes (Figure 3.7 A). This can be explained by a greater decrease in median methylation between HCT116 and 3BKO in this population when compared to CpG island genes, as depicted by the box-and-whisker plots (Figure 3.7 B). Similarly, loss of DNMT3B had a greater effect on global DNA methylation patterns of non-CpG island genes in gene bodies (Figure 3.8 A). Upon further inspection, there is a more significant decrease in minimum methylation values in the 3BKO cells of non-CpG island genes when compared to the CpG island genes that may account for most changes seen (Figure 3.8 B). These results can be attributed to the fact that non-CpG

island genes started with more basal methylation than CpG island genes in both promoter and gene body regions.

We reasoned by examining all probes associated with a particular DNMT3B target gene, that local methylation dynamics across the gene could be more easily discernible. A plot of *SOX9* shows loss of DNMT3B had a minimal effect on the methylation status on this CpG island gene (Figure 3.9 A). At *SI00A3*, a non-CpG island gene, loss of DNMT3B resulted in a specific decrease in methylation (Figure 3.9 B). This includes a selective loss around the TSS which may account for gene expression changes. Overexpression of both wild type and mutant DNMT3B failed to restore methylation at this gene. This result was expected. Normally a longer time frame is required to see DNA methylation changes than the 48 hour period used for this study. To truly assess if these local trends are representative for the larger subclasses, further gene-wide characterization analysis is necessary.

Other data supports the finding that non-CpG island genes experience a specific methylation loss due to DNMT3B. When the sum methylation of 26 non-CpG island genes at promoter regions were compared with 26 genes randomly selected from the genome, the methylation pattern appeared to be specific to DNMT3B target promoters (Figure 3.10). The randomly selected genes started with more median methylation overall and the loss of DNMT3B had a negligible effect. This study supports the notion that DNMT3B influences global DNA methylation patterns according to CpG status. Methylation of non-CpG island genes were affected by loss of DNMT3B. However, DNMT3B did not seem to play a significant role in methylation of CpG island genes, namely at promoters.

DNMT3B is recruited back to endogenous target gene promoters

In order to better understand the mechanism of repression at CpG island gene loci, chromatin immunoprecipitation was performed. The promoter region of DNMT3B target genes showed a loss of DNMT3B in the 3BKO setting but increased enrichment for DNMT3B after overexpression of wild-type DNMT3B (Figure 3.11 A). LSD1 is a histone modifying enzyme known to function as a histone demethylase and transcriptional corepressor [11]. Recruitment of LSD1 mirrored that of DNMT3B (Figure 3.11 B). In support of our hypothesis, this result suggests other repressive epigenetic proteins can occupy promoters of DNMT3B target genes. Ongoing studies are being performed to determine if mutant DNMT3B can also be recruited back to target promoters. Future experiments include studying if these proteins directly interact or occur in a larger repressive complex.

DNMT3B recruitment alters histone modifications at endogenous target gene promoters

When H3K4me3 is localized to promoter regions it correlates with transcriptional activation. Another histone modification, H3K27me3, is associated with a repressive chromatin state. Genes that are marked by both are considered bivalent. Bivalent chromatin has both activating and repressive epigenetic modifications in the same vicinity. We were curious to assess the chromatin status of DNMT3B target genes in the presence and absence of DNMT3B. Loss of DNMT3B correlated with increased H3K4me3 at target genes and both wild-type and mutant DNMT3B depleted this mark (Figure 3.12). DNMT3B has been associated with genes that harbor repressive

H3K27me3 modifications [12]. However, in our study H3K27me3 remain relatively unchanged (Figure 3.12).

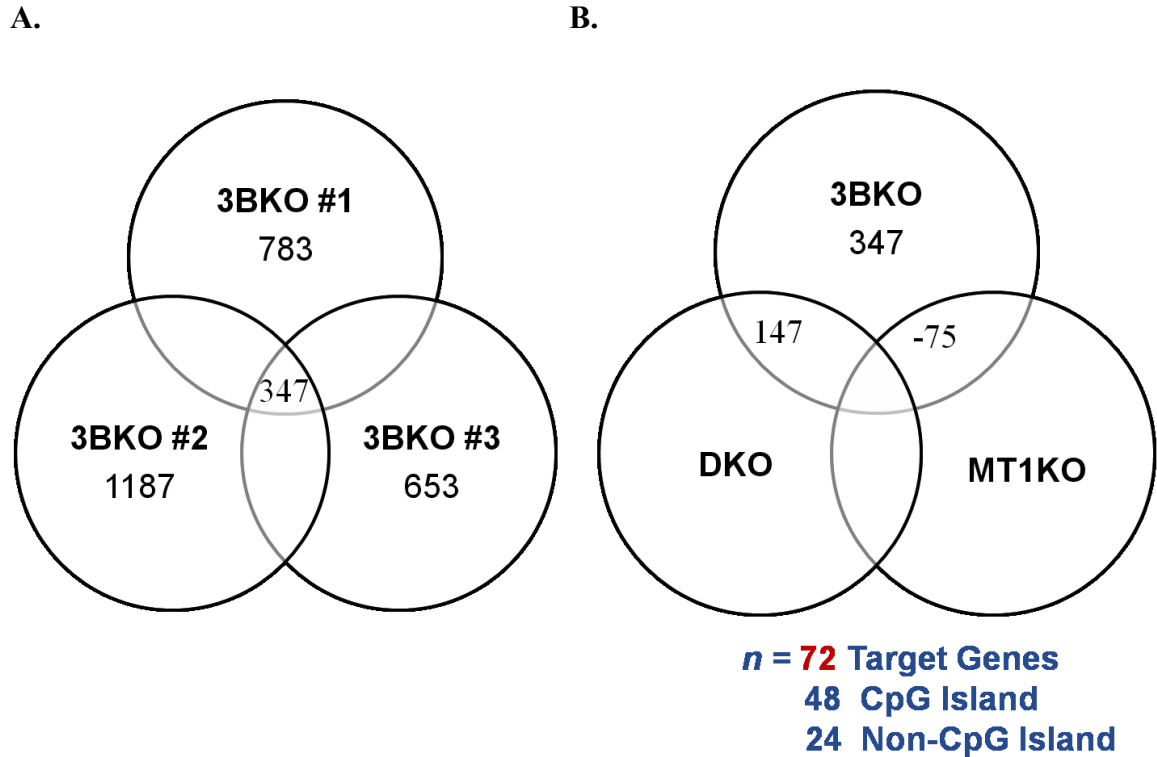
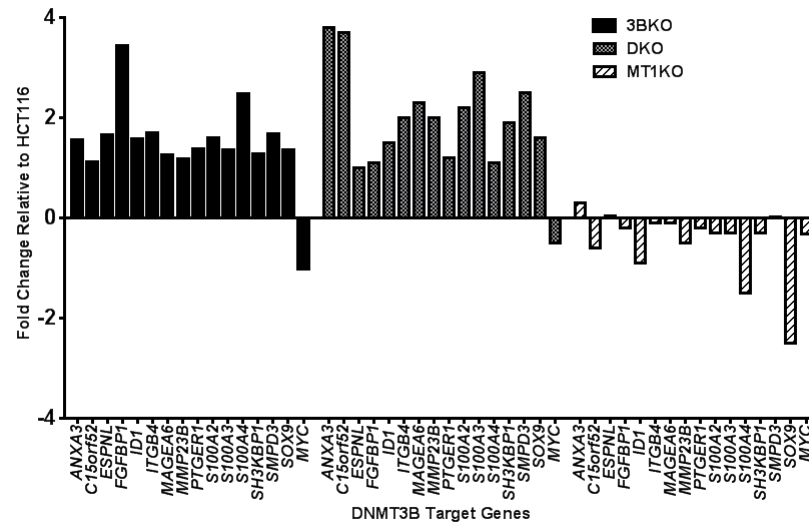


Figure 3.1. Statistical filtering of DNMT3B candidate genes. (A) A total of 347 up-regulated genes were found in common between stable clones containing an empty vector (3BKO #1, 3BKO #2, and 3BKO #3) in a *DNMT3B*^{-/-} genetic knockout background compared to parental HCT116. All 347 genes shared have a normalized log differential expression ratio of ≥ 0.5 . (B) A total of 147 genes were up-regulated in all three 3BKO clones and a DKO clone that lacks both *DNMT3B*^{-/-} and full length *DNMT1*^{-/-}. Cross reference analysis excluded 75 genes up-regulated in a *DNMT1*^{-/-} hypomorph clone. After exclusion of DNMT1 target genes, 72 DNMT3B candidate genes were selected for further analysis. All comparisons were made using a Whitehead Institute for Biomedical Research Bioinformatics and Research Computing comparison tool (<http://jura.wi.mit.edu/bioc/tools/compare.php>).

A.



B.

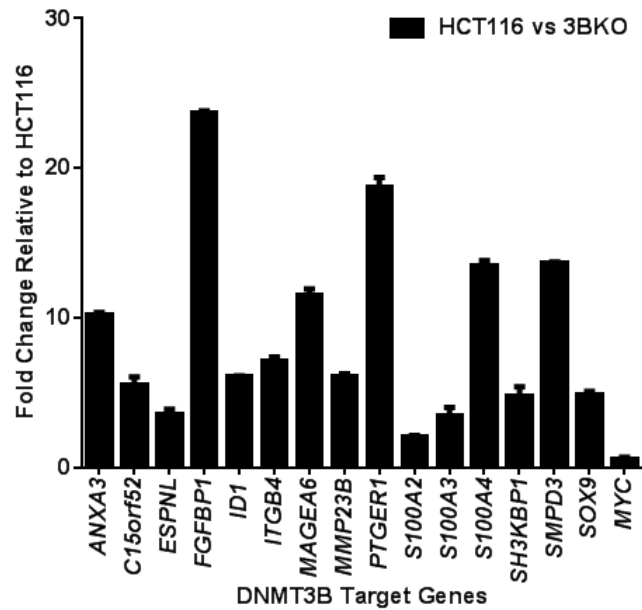


Figure 3.2. Genetic disruption of DNMT3B in parental HCT116. (A) Microarray expression data in various DNMT knockout settings. (B) Quantitative RT-PCR candidate gene validation in 3BKO clones compared to parental HCT116. *MYC*, a gene not under the control of DNMT3B serves as a control. Error bars represent the standard error of the mean for 3 independent biological experiments.

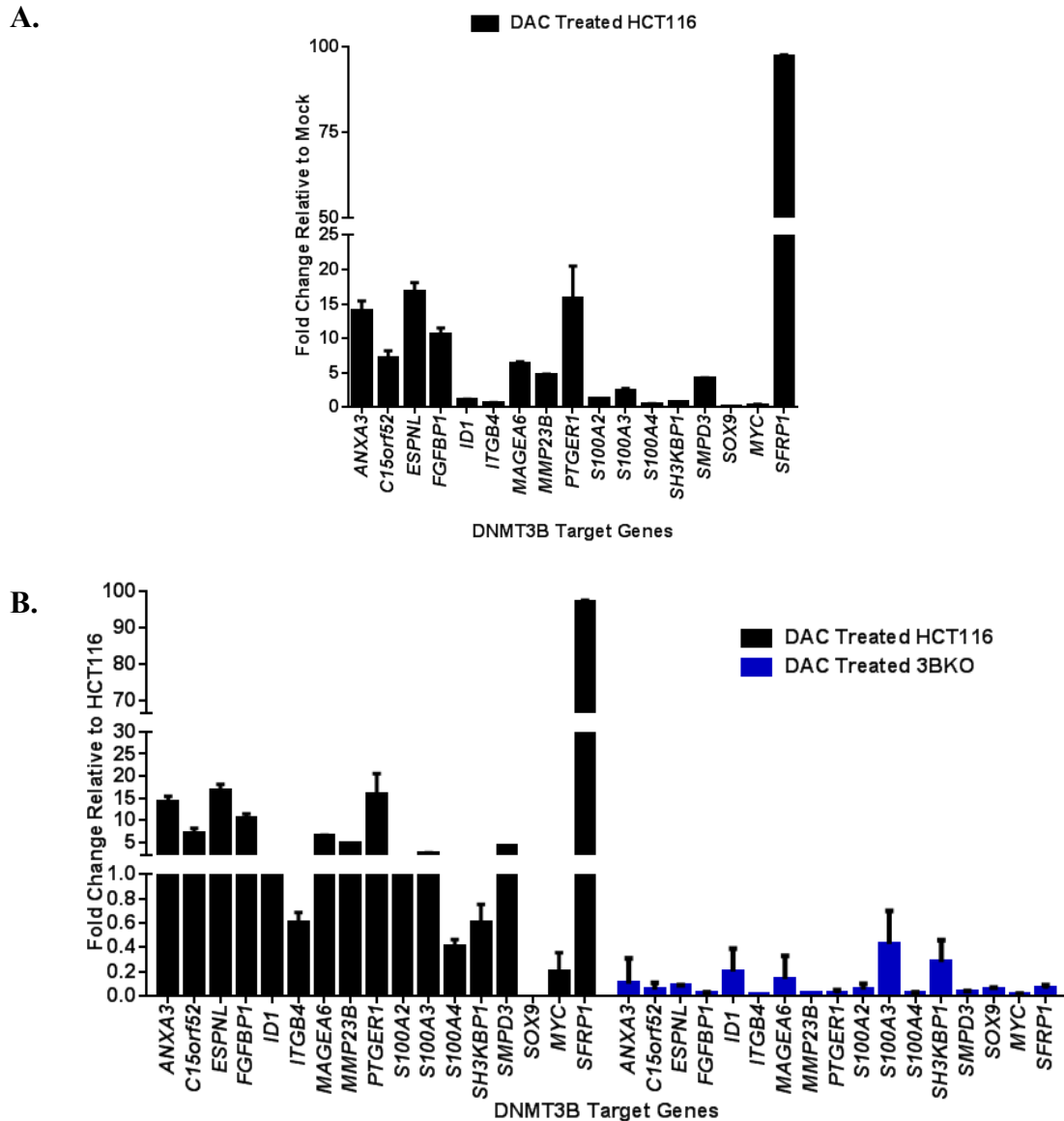


Figure 3.3. Pharmacologic inhibition of DNMTs in parental HCT116 and 3BKO cells. (A) Quantitative RT-PCR candidate gene expression after a 500 nM DAC treatment for 72 hours in HCT116 cells. (B) Quantitative RT-PCR candidate gene expression after a 500 nM DAC treatment for 72 hours in 3BKO cells. Samples were then normalized to baseline gene expression in parental HCT116. PBS served as the mock negative control and *SFRP1* was used as a positive control for drug efficacy. Error bars represent the standard error of the mean for 3 independent biological experiments.

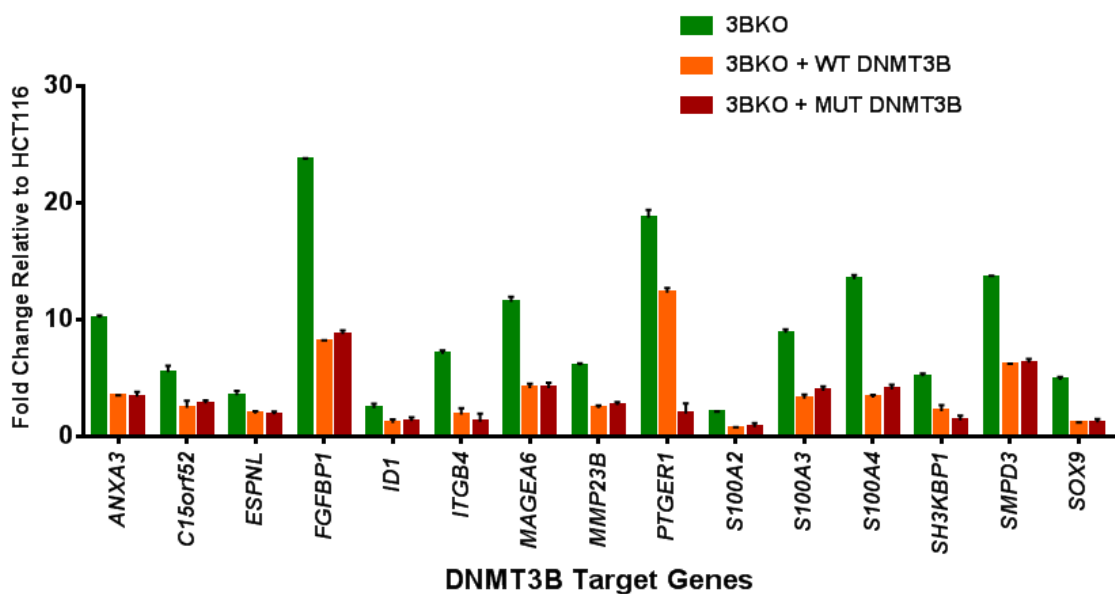


Figure 3.4. The effect of wild type and mutant DNMT3B on target gene expression.

Quantitative RT-PCR candidate gene expression after overexpression of wild type and mutant DNMT3B for 48 hours in 3BKO cells. Samples were then normalized to baseline gene expression in parental HCT116. Error bars represent the standard error of the mean for 3 independent biological experiments.

A.

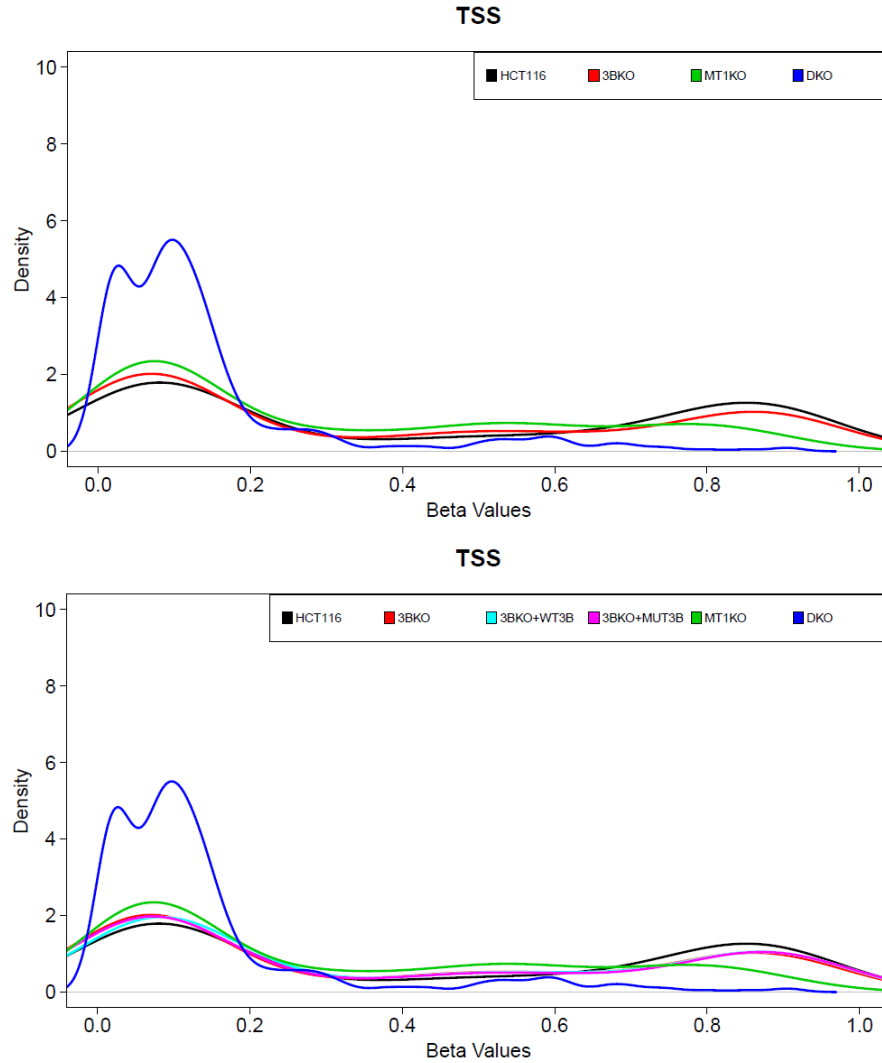


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

B.

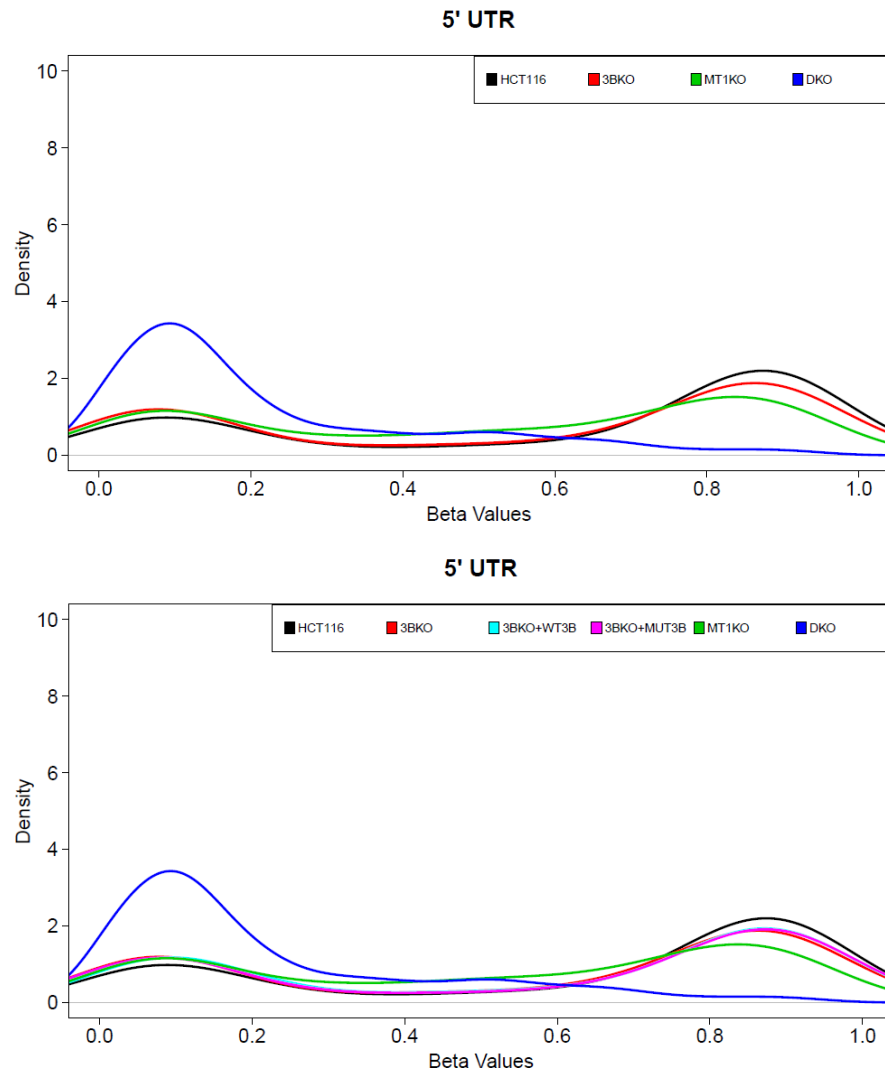


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

C.

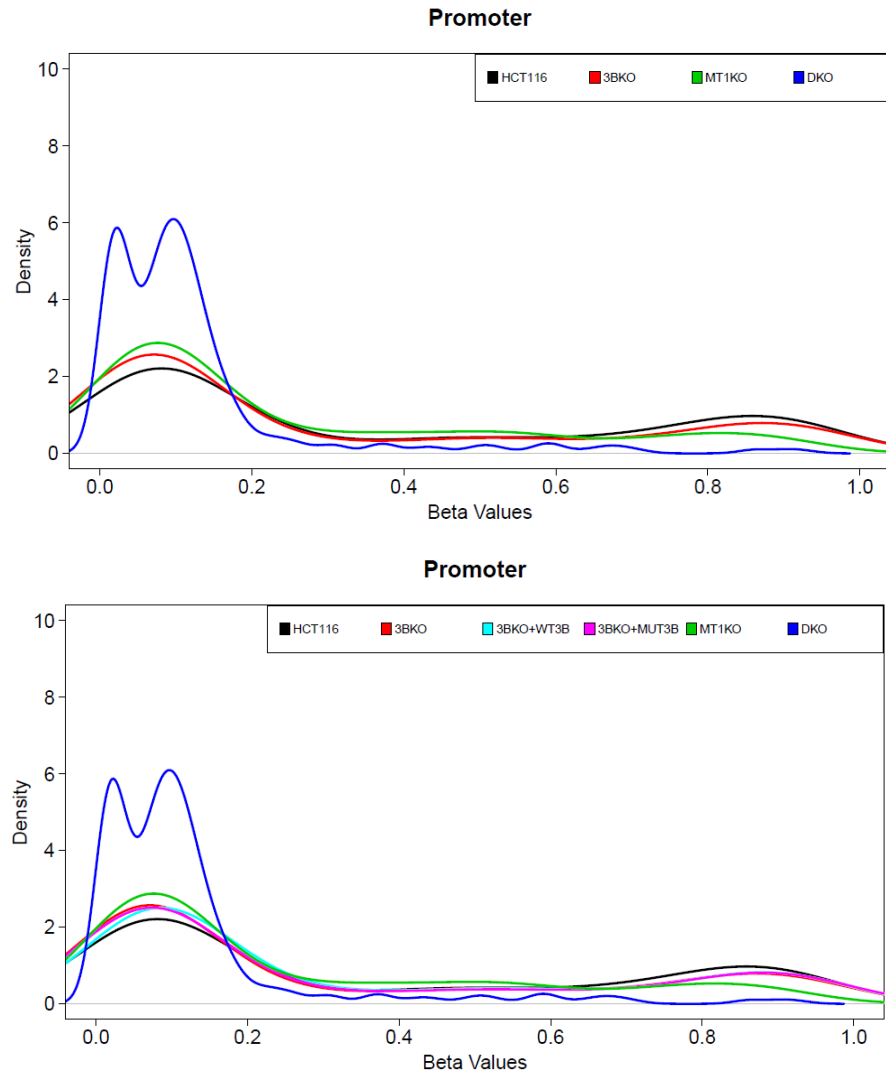


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

D.

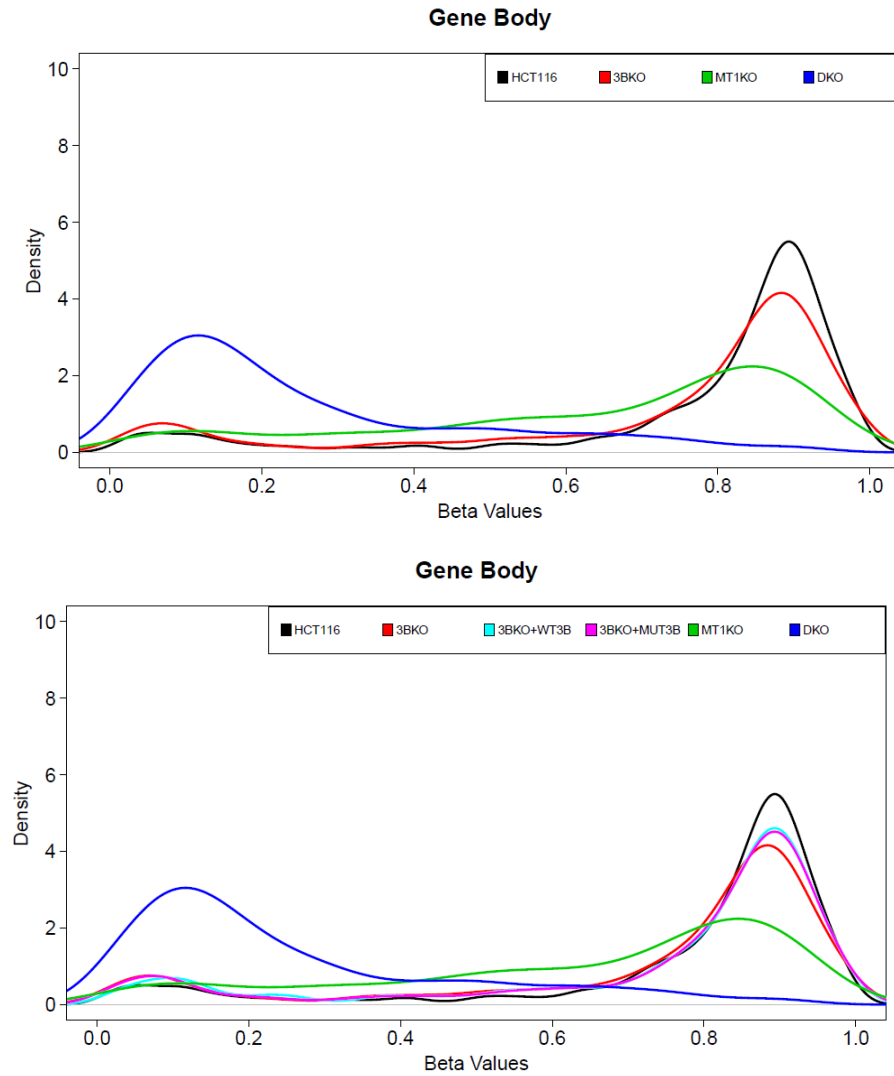


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

E.

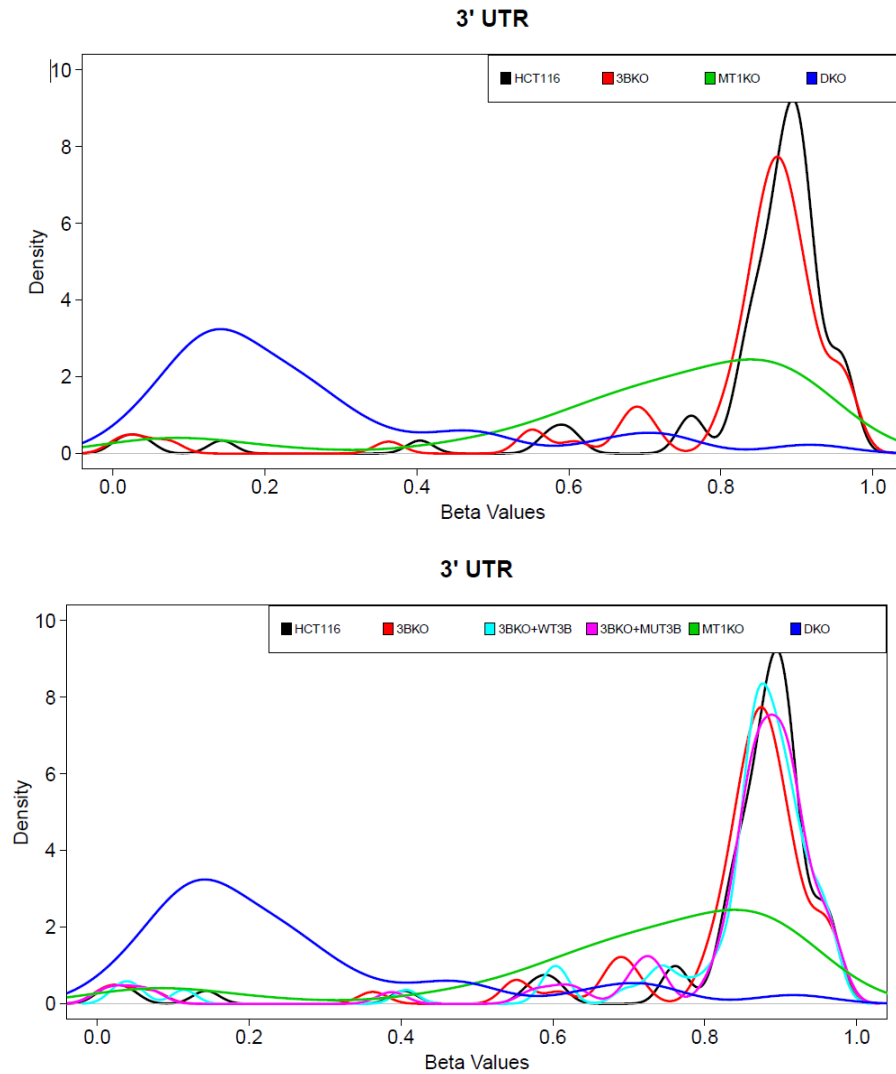


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

F.

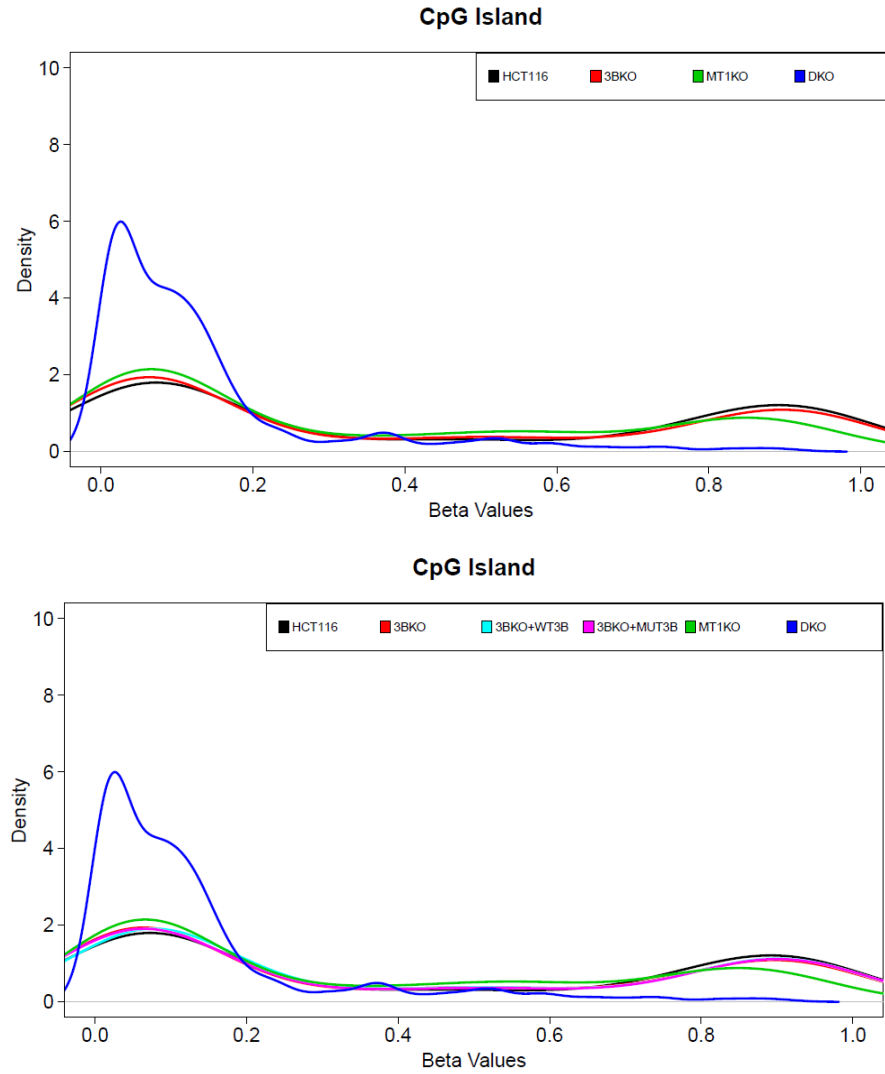


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

G.

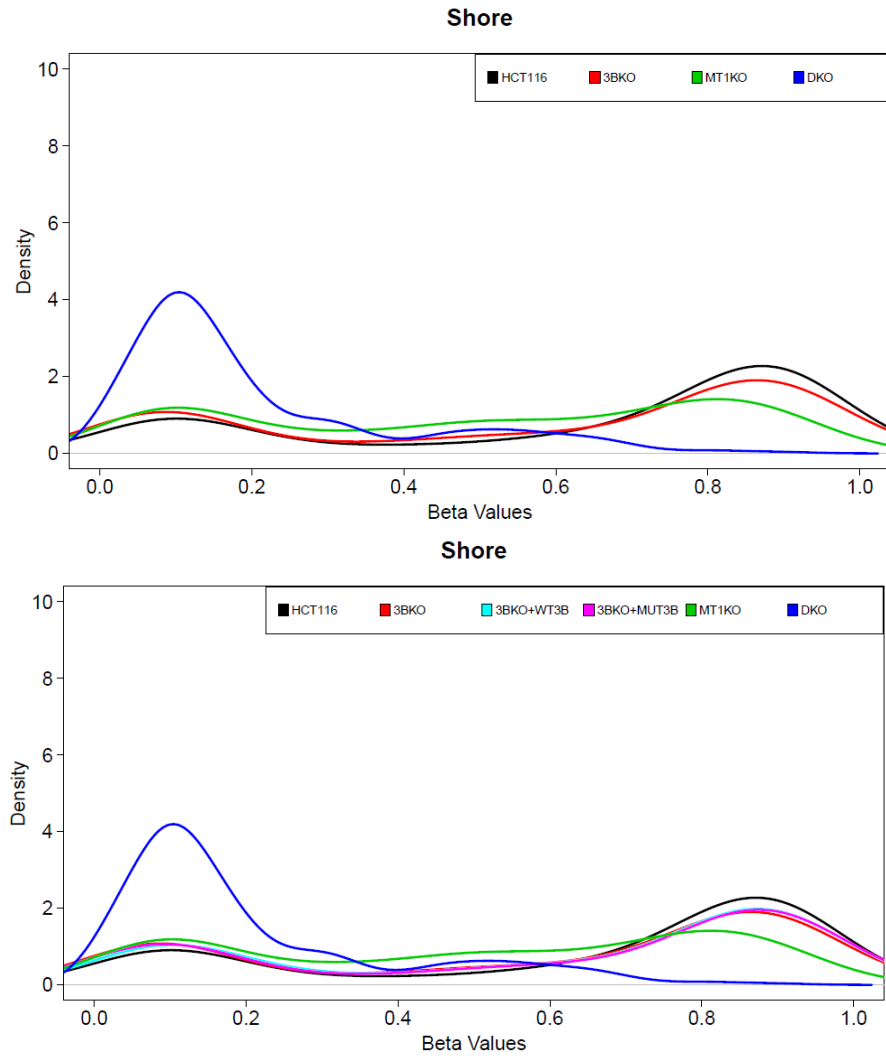


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

H.

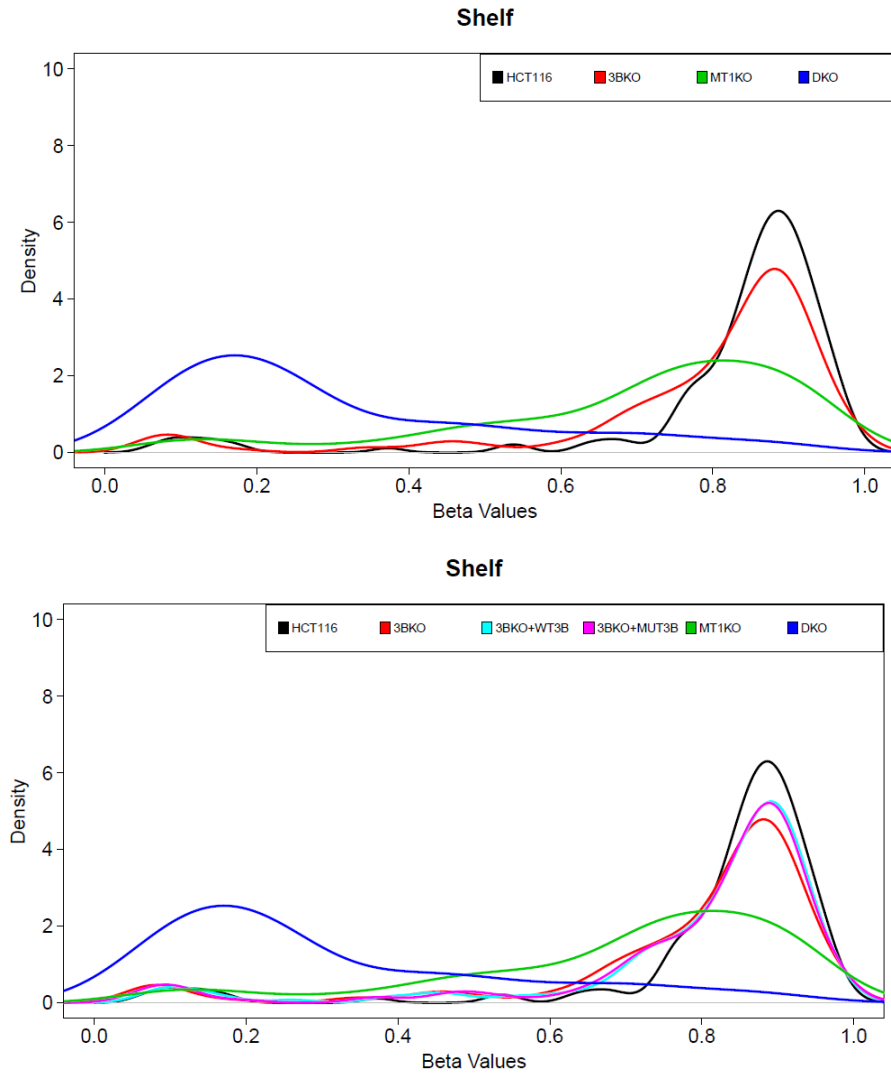
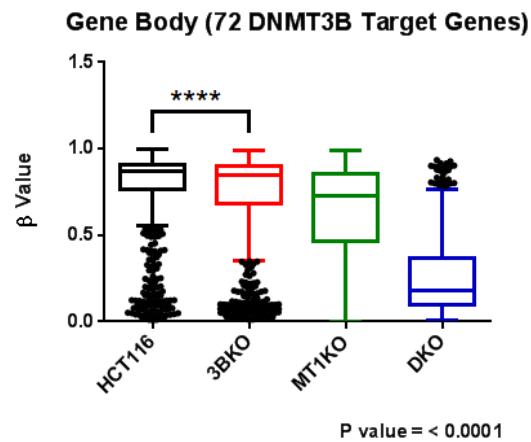
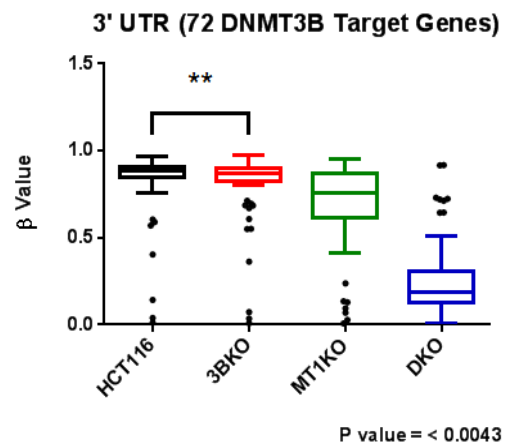


Figure 3.5. Global distribution of DNA methylation upon loss and restoration of DNMT3B according to genomic location. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation (hypomethylation) and β values closer to 1 reflect the most abundant methylation (hypermethylation) at various genomic regions; (A) TSS (B) 5' UTR (C) Promoter (D) Gene Body (E) 3' UTR (F) CpG Islands (G) Shore (H) Shelf.

A.



B.



C.

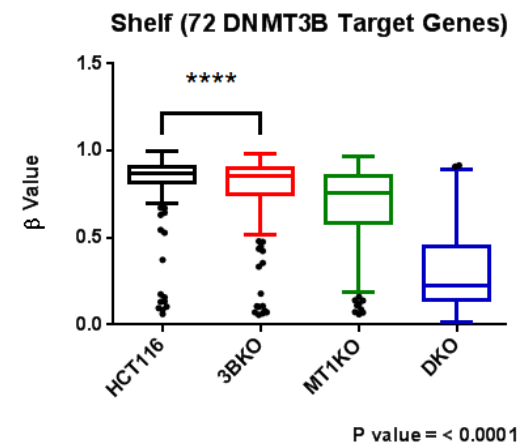
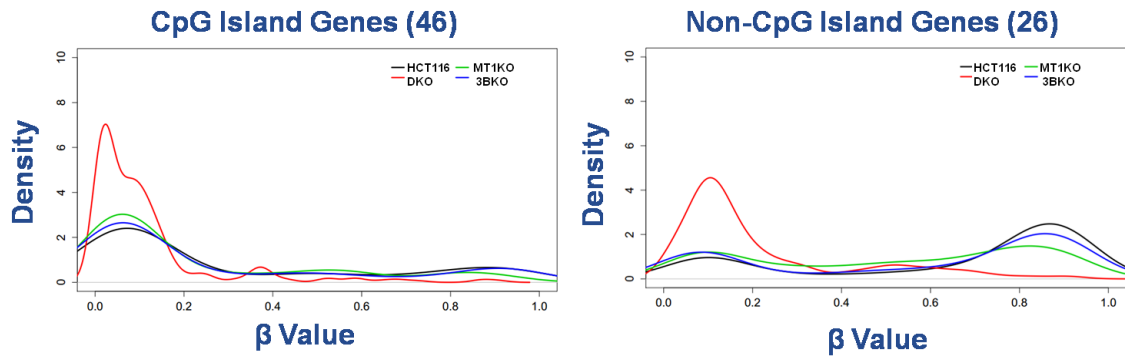


Figure 3.6. Paired comparison of methylation in parental HCT116 relative to 3BKO cells. A two-tailed paired t-test was used to assess statistical significance of differentially methylated regions; (A) Gene Body (B) 3' UTR (C) Shelf.

A.



B.

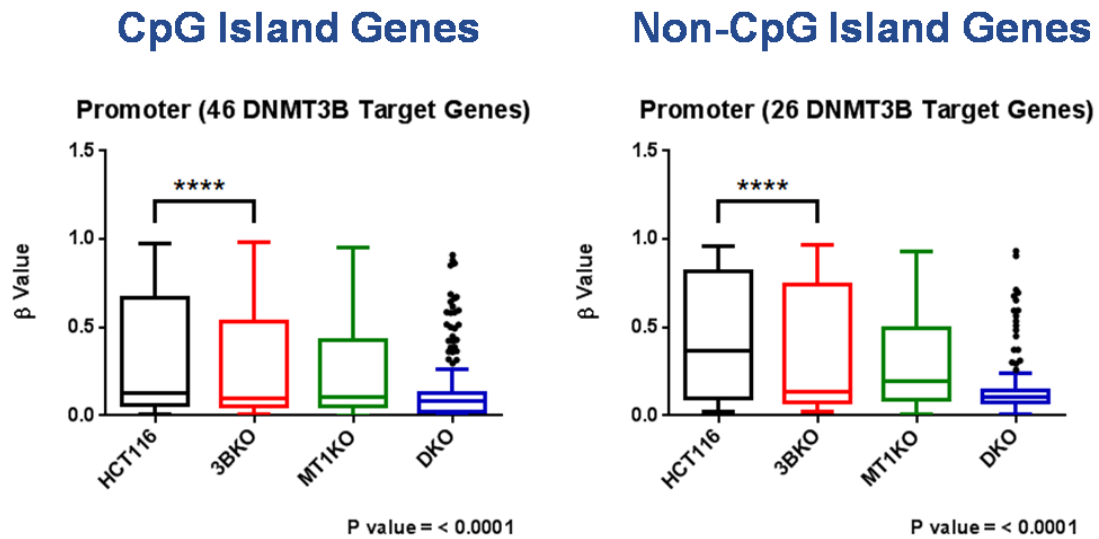
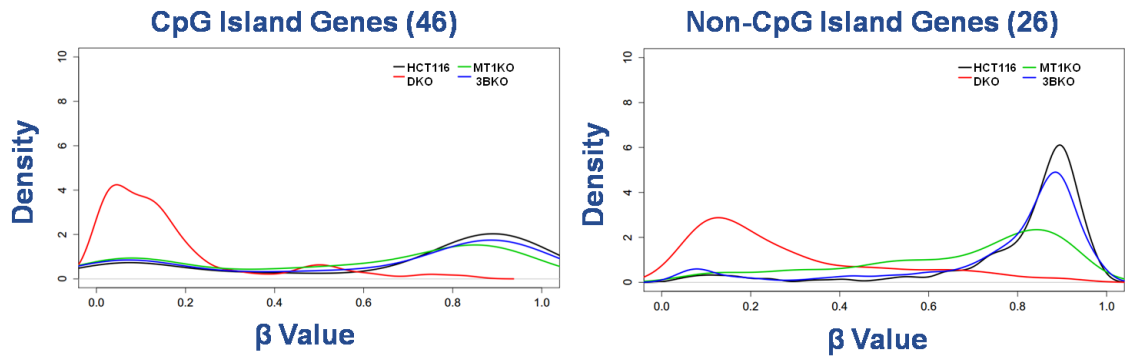


Figure 3.7. Methylation patterns according to CpG status at promoters. (A) Global distribution of DNA methylation upon loss and restoration of DNMT3B. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation and β values closer to 1 reflect the most abundant methylation) at a genomic region known to regulate transcriptional control; (Left) CpG island genes (Right) Non-CpG island genes (Black = HCT116, Blue = 3BKO, Green = MT1 hypomorph, Red = DKO). (B) Paired comparisons of methylation in parental HCT116 relative to 3BKO cells according to CpG island status.

A.



B.

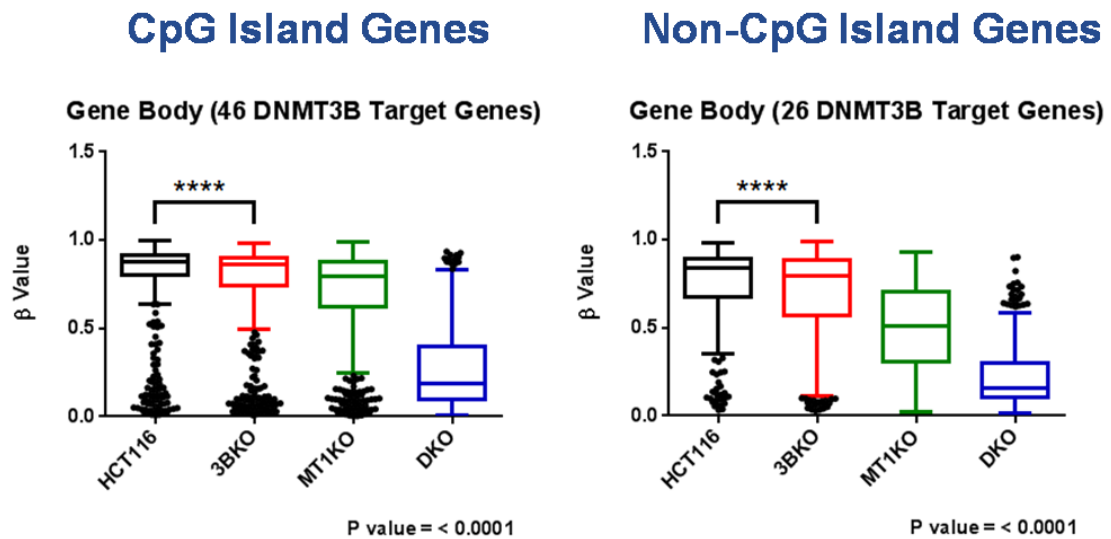
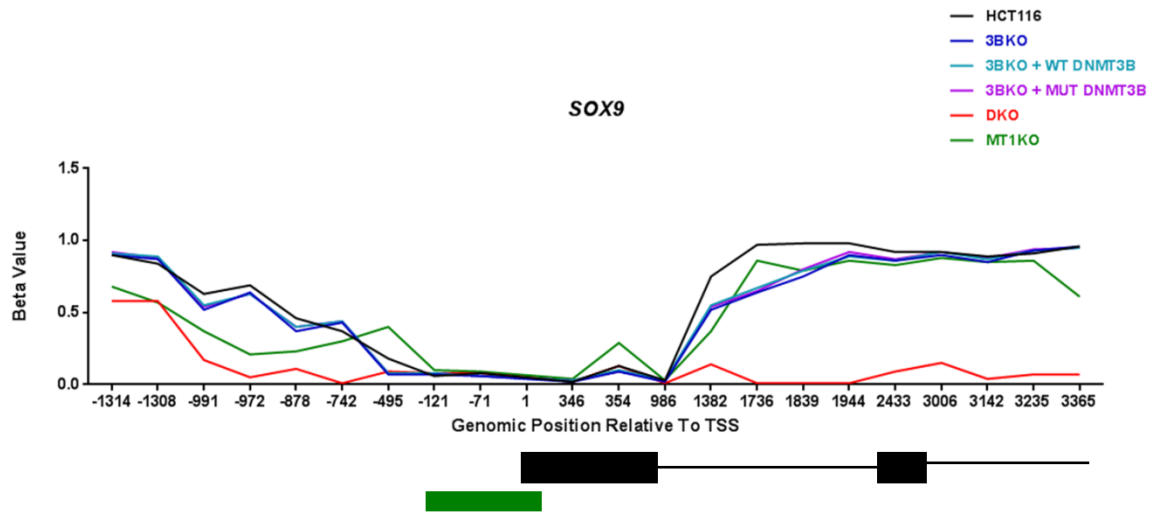


Figure 3.8. Methylation patterns according to CpG status at gene bodies. (A) Global distribution of DNA methylation upon loss and restoration of DNMT3B. Kernel density plots depicting methylation values along the x-axis (β values closer to 0 reflect the least methylation and β values closer to 1 reflect the most abundant methylation) at a genomic region implicated in transcriptional control; (Left) CpG island genes (Right) Non-CpG island genes (Black = HCT116, Blue = 3BKO, Green = MT1 hypomorph, Red = DKO). (B) Paired comparisons of methylation in parental HCT116 relative to 3BKO cells according to CpG island status.

A.



B.

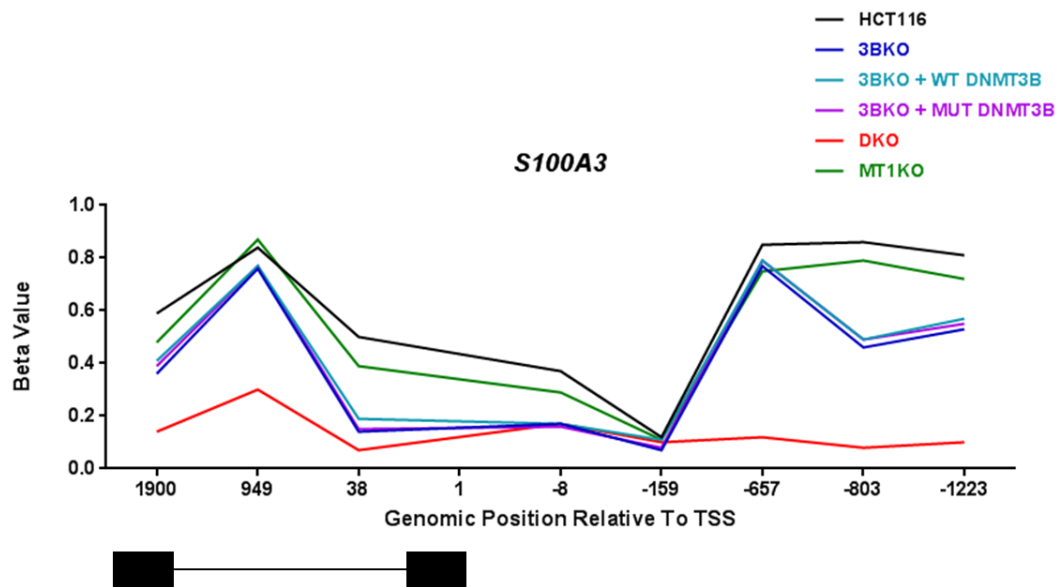


Figure 3.9. Representative plots of methylation across DNMT3B target genes. (A) CpG island gene (B) non-CpG island gene (Black boxes = exons, Green boxes = CpG island)

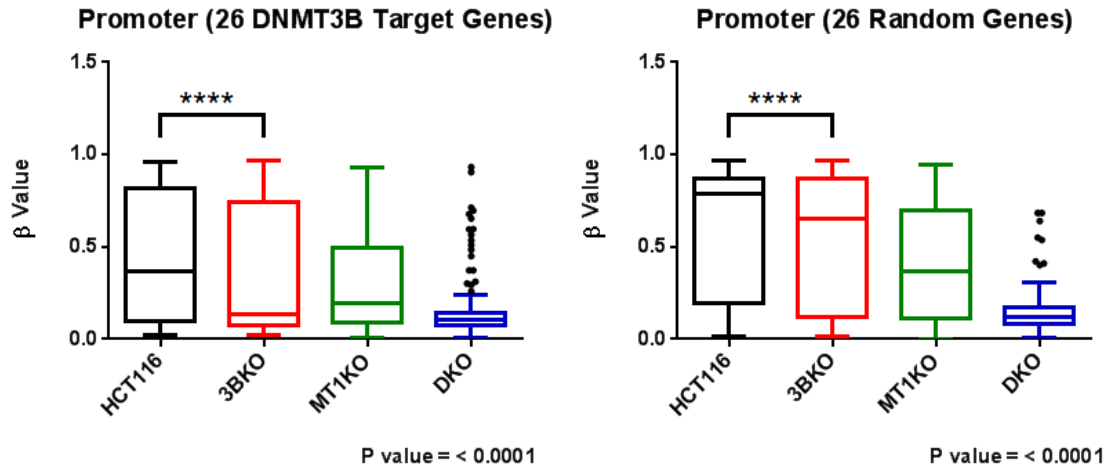
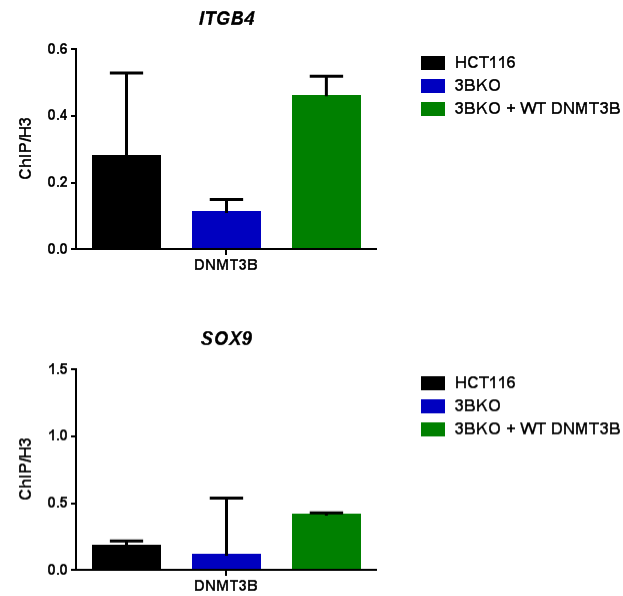


Figure 3.10. A methylation comparison between non-CpG island DNMT3B targets and random genes. (Left) 26 non-CpG island DNMT3B target genes (B) 26 non-CpG island genes randomly selected from the genome.

A.



B.

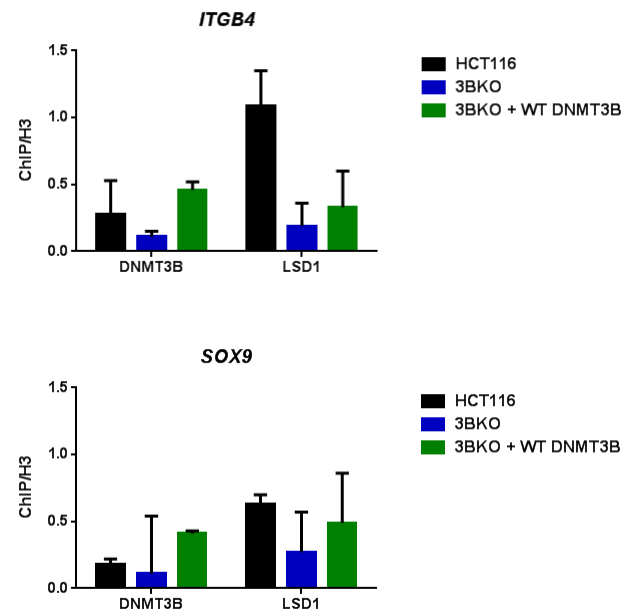


Figure 3.11. Repressive protein recruitment to DNMT3B target genes. Analysis of *ITGB4* and *SOX9* promoter regions. Error bars denote standard error of the mean for biological duplicates. (A) DNMT3B enrichment (B) LSD1 enrichment

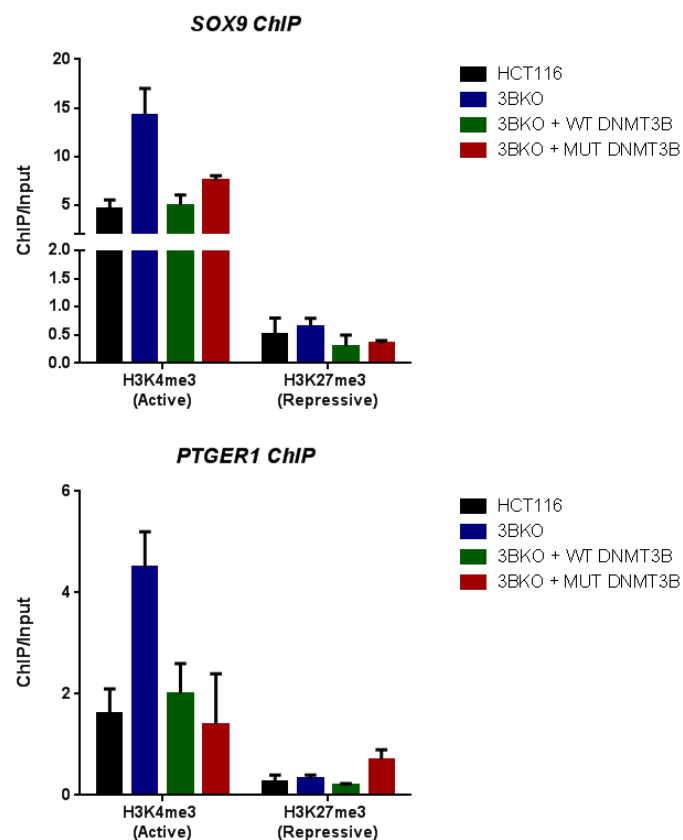


Figure 3.12. Histone modifications at DNMT3B target genes. Analysis of *ITGB4*, *SOX9*, and *PTGER1* promoter regions. Error bars denote standard error of the mean for biological duplicates.

Table 3.1 qRT-PCR Primer Sequences

<i>ANXA F</i>	CTG GGG CCG CAA TCA GGT GG
<i>ANXA R</i>	TGC GAG CGA AGG TGT CAG CC
<i>C15orf52 F</i>	CCG CGT GAT GGC CAT GTG GT
<i>C15orf52 R</i>	GGG AGG CCA CTC CTT GCC CT
<i>ESPNL F</i>	AGA GTG GAA GCG GCA GGT GAT G
<i>ESPNL R</i>	CCT GAT GAG TCT GTG AGT ACC TC
<i>FGFBP1 F</i>	CCC AGT CAG CCT GGC TCC TGT T
<i>FGFBP1 R</i>	CAT GGC TGC AGC TGG GCG TT
<i>ID1 F</i>	CCG CAA GGT GAG CAA GGT GGA G
<i>ID1 R</i>	CAG GAA CGC ATG CCG CCT CG
<i>ITGB4 F</i>	CCC AGA GCG GGG AGG ACT ACG
<i>ITGB4 R</i>	CTC GAA CTT CCA GCC GCA GCC
<i>MAGEA6 F</i>	CCA GGC TCG GTG AGG AGG CA
<i>MAGEA6 R</i>	CAG GGC AAC AGG CGG GAG TG
<i>MMP23B F</i>	AAG GCG TGT GGC TCA CGG AC
<i>MMP23B R</i>	AGC GTG GCG TTC AGG TGC AT
<i>PTGER1 F</i>	CGC TGG TGT GCA ACA CGC TC
<i>PTGER1 R</i>	GGC CGA AGC GAT GGA CGA GG
<i>S100A2 F</i>	GTC CAG GAT GCC CAG TCC CCA
<i>S100A2 R</i>	TCC CTC CCA GAG AGT GCC AGC
<i>S100A3 F</i>	TCG GGG GCT GGG CAT GTC TC
<i>S100A3 R</i>	GGG CCC CAG AGT GGC TCT ACC
<i>S100A4 F</i>	TCT TCC CCT CTC TAC AAC CCT CTC T
<i>S100A4 R</i>	CAT GAC AGC AGT CAG GAT CAA ACC A
<i>SH3KBP1 F</i>	ACG GCA GGA GAG GTT TGT TCC CT
<i>SH3KBP1 R</i>	GAA TGC CAC CTG GCA CCG GC
<i>SMPD3 F</i>	GAA GCA CAC CTC AGG ACC AAA G
<i>SMPD3 R</i>	CAG CCA GTC CTG AAG CAG GTC
<i>SOX9 F</i>	GTG CAA GCG CCC CCA CTT TT
<i>SOX9 R</i>	GCT ACC GCG GCG AGC ACT TA

Table 3.2 ChIP Primer Sequences

<i>ITGB4 F1</i>	GAGCTCCTGCTCCATGTTTC
<i>ITGB4 R1</i>	GGAGTCCCAGTTTGGTTGAA
<i>SOX9 F</i>	GCTCGCTCCAGGCGCGTAAA
<i>SOX9 R</i>	CACTGCTCTCTCCGCGGCAC
<i>PTGER1 F</i>	GGGGGTCACATGGGGCCAGA
<i>PTGER1 R</i>	CCCCCTCTCTGCTCCTGGCA

Discussion

In this study we report at some endogenous target genes, DNMT3B acts as a transcriptional co-repressor independent of its catalytic activity.

Previous work from our lab and others has shown DNMT3b can repress transcription of reporter genes and that the N-terminal domain alone is sufficient to facilitate gene repression [3, 13]. The repressive effects of DNMT3B does not require enzymatic activity and can be mediated through HDACs [7]. The drawback to such studies is it prevents the study of chromatin dynamics at endogenous loci. We found that enzymatic activity of DNMT3B was dispensable for transcriptional silencing. These changes were associated with loss of DNA methylation at non-CpG island genes. By contrast, methylation changes were negligible at CpG island genes. At the promoter of some CpG island genes we witnessed an increase in active histone marks while repressive histone marks remained relatively unchanged. Gene repression can be mediated through an ATRX-like region known as the PHD domain, which is found in many chromatin associated proteins. The PHD domain is absent in DNMT1 and only found in the DNMT3 protein family. HDAC1 has been reported to directly bind proteins at their PHD regions [14]. DNMT3L, which interacts with DNMT3B, lacks a C-terminal catalytic domain but still exerts transcriptional repression through associations with HDACs [14, 15]. In fact, DNMT3B is known to make multiple interactions with HDACs [3, 4, 7]. It is possible the PHD domain facilitated the repression at DNMT3B target genes identified in this investigation. Further work will address HDAC recruitment to DNMT3B targets.

Acknowledgements

Dr. Joo Mi Yi assisted with cloning and helpful discussions about cloning strategies. Dr. Eriko G. Clements provided useful discussions in regards to project ideas and experimental design. Dr. Hariharan Easwaran and Muhammad Noon helped with the DNA methylation and gene expression microarray analyses. Dr. Heather O'Hagan offered technical advice for ChIP studies. Dr. Heather O'Hagan and Dr. Robert Casero both contributed helpful constructive feedback in the preparation of this manuscript.

References

1. Van Emburgh, B.O. and K.D. Robertson, *Modulation of Dnmt3b function in vitro by interactions with Dnmt3L, Dnmt3a and Dnmt3b splice variants*. Nucleic Acids Res, 2011. 39(12): p. 4984-5002.
2. Clements, E.G., et al., *DNMT1 modulates gene expression without its catalytic activity partially through its interactions with histone-modifying enzymes*. Nucleic Acids Res, 2012. 40(10): p. 4334-46.
3. Bachman, K.E., M.R. Rountree, and S.B. Baylin, *Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin*. J Biol Chem, 2001. 276(34): p. 32282-7.
4. Bai, S., et al., *DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2*. Mol Cell Biol, 2005. 25(2): p. 751-66.
5. Boland, M.J. and J.K. Christman, *Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA*. J Mol Biol, 2008. 379(3): p. 492-504.
6. O'Hagan, H.M., et al., *Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG Islands*. Cancer Cell, 2011. 20(5): p. 606-19.
7. Rhee, I., et al., *DNMT1 and DNMT3b cooperate to silence genes in human cancer cells*. Nature, 2002. 416(6880): p. 552-6.
8. Rhee, I., et al., *CpG methylation is maintained in human cancer cells lacking DNMT1*. Nature, 2000. 404(6781): p. 1003-7.

9. Bibikova, M., et al., *High density DNA methylation array with single CpG site resolution*. Genomics, 2011. 98(4): p. 288-95.
10. Jeltsch, A., *Molecular enzymology of mammalian DNA methyltransferases*. Curr Top Microbiol Immunol, 2006. 301: p. 203-25.
11. Shi, Y., et al., *Histone demethylation mediated by the nuclear amine oxidase homolog LSD1*. Cell, 2004. 119(7): p. 941-53.
12. Choi, S.H., et al., *Identification of preferential target sites for human DNA methyltransferases*. Nucleic Acids Res, 2011. 39(1): p. 104-18.
13. Fuks, F., et al., *Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription*. EMBO J, 2001. 20(10): p. 2536-44.
14. Aapola, U., I. Liiv, and P. Peterson, *Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity*. Nucleic Acids Res, 2002. 30(16): p. 3602-8.
15. Deplus, R., et al., *Dnmt3L is a transcriptional repressor that recruits histone deacetylase*. Nucleic Acids Res, 2002. 30(17): p. 3831-8.

Chapter 4
Conclusions and Future Directions

The overarching goal of this work was to explore epigenetic mechanisms responsible for DNA hypermethylation and gene silencing in cancer. We addressed this question by attempting to understand (1) which genomic regions are controlled by DNMT3B-mediated DNA methylation and (2) what epigenetic mechanisms govern transcriptional repression at DNMT3B target genes after recruitment to endogenous promoters.

DNA methylation and common regulatory mechanisms are conserved amongst most eukaryotic groups, including plants, animals, and insects [1-5]. Under normal circumstances, centromeric and repetitive DNA are heavily methylated, promoter regions lack methylation, and gene body regions show high levels of methylation. Recent studies have specifically implicated the DNMT3 family members as key in modulating DNA methylation outside of the promoter in all three model organisms [1, 3]. Due to the growing body of literature, we were most curious about the role of DNMT3s in gene body methylation.

Cancer is hallmarked by aberrant methylation patterns and dysregulation of DNA methylation. For our first aim, we tested the hypothesis that DNMT3B may regulate DNA methylation patterns at non-promoter genomic regions in cancer. By comparing HCT116 (a colorectal cancer cell line) with a derivative cell line 3BKO (genetically devoid of both alleles of DNMT3B), we queried 8 genomic signatures for changes in DNA methylation. Four regions displayed a statistically significant loss of DNA methylation upon DNMT3B removal, and all signatures were distal to the promoter (shore, shelf, gene body, and 3' UTR). Overexpression of wild type DNMT3B, but not catalytically dead DNMT3B, resulted in a slight gain of methylation. Our data agrees

with other labs that gene body regions of cancer cells can retain significant amounts of DNA methylation [6]. The fact that DNMT3B seems to somehow influence DNA methylation in this region the most raises interesting questions about CpG methylation outside of the promoter CpG island context. It is possible that DNMT3B is normally bound to these regions. ChIP-Seq studies are the next logical steps towards understanding DNMT3B targeting. In specific, this study can be extended further by comparing the localization of both wild type and mutant DNMT3B.

Prevailing theory states mammalian DNA methylation occurs in the CpG dinucleotide context. However, limited studies have revealed significant quantities of non-CpG methylation in the genome [7, 8]. Non-CpG methylation is defined as CpA, CpT, and CpC. Such non-CpG methylation was found to be more prevalent in human embryonic stem cells (15-20%) as opposed to somatic tissues (negligible) [7-9]. The functional significance of non-CpG methylation is still undetermined.

DNMT1 affected CpG methylation, while Dnmt3a induced CpG, CpA, and CpT methylation *in vivo* using a transgenic *Drosophila* model [8]. Due to sequence similarities, Dnmt3b was not studied. To date, no genome-wide non-CpG assay has been performed looking at DNMT3B targeting. CpG methylation occurs on both strands in mammals. However, non-CpG methylated DNA is hemi-methylated. *De novo* methyltransferase activity may explain preferential methylation of non-CpG DNA after replication. Research has shown non-CpG methylation disappears during differentiation and reappears in induced pluripotent stem cells [7]. For future work, it would be of great interest to study how global non-CpG methylation patterns vary in the presence and absence of DNMT3B. Non-CpG methylation has been associated with a stem-cell

phenotype. Cancers gain hypermethylation at genes that are unmethylated in stem cells, have bivalent chromatin, and are reversibly repressed by PRCs. Work from our lab has contributed to a model of a stem/progenitor cell signature in cancers [10, 11]. DNMT3B could provide interesting new functional clues about this non-traditional methylation in cancer.

Work has also been done to identify links between non-CpG methylation (around the TSS and in the gene body) and gene expression. Similar to CpG methylation, non-CpG methylation decreased around the TSS and returned to a normal density ~2 kb upstream near the 5' UTR. In exons, introns, and 3' UTRs, non-CpG methylation was twice as high [7]. At gene bodies, higher expressed genes had 3 times the amount of non-CpG methylation than genes not expressed [7].

We have proven DNMT3B has a distinct set of endogenous target genes in colorectal cancer cells and both wild-type and mutant DNMT3B repress target genes. DNA methylation at CpG island promoters is not required for this gene repression. However, this may be a causative mechanism in non-CpG island genes. DNMT3B and LSD1, histone demethylase, can be recruited back to target genes, raising the possibility that these loci may be co-regulated. Loss of DNMT3B correlates with an increased active histone mark (H3K4me3) at target genes while repressive histone marks remain relatively unchanged (H3K27me3). Regulation of DNMT3B target genes seem to involve multiple mechanisms according to CpG status.

Of note, transiently transfected DNMT3B was tolerated in the somatic cancer cell lines used in this study but a stably integrated DNMT3B transgene was not. This suggests tight regulation of DNMT3B by cells. Perhaps to prevent further hypermethylation or

disrupt regulatory mechanisms allowing for translational control. DNMT3B is also known to be selectively expressed in some tissues and has many splice variants in cancer. It would be interesting to further investigate how splice variants influence the DNA methylation patterns identified in various genomic regions. This would refine the molecular underpinning of which DNMT3B domains are necessary for the regulation of global and local methylation patterns.

References

1. He, X.J., T. Chen, and J.K. Zhu, *Regulation and function of DNA methylation in plants and animals*. Cell Res, 2011. **21**(3): p. 442-65.
2. *Insights into social insects from the genome of the honeybee Apis mellifera*. Nature, 2006. **443**(7114): p. 931-49.
3. Kucharski, R., et al., *Nutritional control of reproductive status in honeybees via DNA methylation*. Science, 2008. **319**(5871): p. 1827-30.
4. Zemach, A., et al., *Genome-wide evolutionary analysis of eukaryotic DNA methylation*. Science, 2010. **328**(5980): p. 916-9.
5. Feng, S., et al., *Conservation and divergence of methylation patterning in plants and animals*. Proc Natl Acad Sci U S A, 2010. **107**(19): p. 8689-94.
6. Varley, K.E., et al., *Dynamic DNA methylation across diverse human cell lines and tissues*. Genome Res, 2013. **23**(3): p. 555-67.
7. Lister, R., et al., *Human DNA methylomes at base resolution show widespread epigenomic differences*. Nature, 2009. **462**(7271): p. 315-22.
8. Ramsahoye, B.H., et al., *Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a*. Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5237-42.
9. Hogart, A., et al., *Genome-wide DNA methylation profiles in hematopoietic stem and progenitor cells reveal overrepresentation of ETS transcription factor binding sites*. Genome Res, 2012. **22**(8): p. 1407-18.

10. Ohm, J.E., et al., *A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing*. Nat Genet, 2007. **39**(2): p. 237-42.
11. Easwaran, H., et al., *A DNA hypermethylation module for the stem/progenitor cell signature of cancer*. Genome Res, 2012. **22**(5): p. 837-49.

Curriculum Vitae

KHADIJAH A. MITCHELL
SCIENTIST · STEM EDUCATOR · SOCIAL ENTREPRENEUR

DATE OF BIRTH: June 27, 1981

LOCATION OF BIRTH: Pittsburgh, PA

HOME

The Belvedere
1 East Chase Street, Suite 603
Baltimore, MD, 21202
412-443-3754
khadijahamitchell@gmail.com

WORK

Sidney Kimmel Comprehensive Cancer Center
1650 Orleans Street, Suite 541
Baltimore, MD, 21231
410-955-8506
kmitch30@jhmi.edu

EDUCATION

7/2014 Johns Hopkins University, Baltimore, MD
Ph.D., Human Genetics and Molecular Biology
(Johns Hopkins School of Medicine)
Certificate, Health Disparities & Health Inequality
(Johns Hopkins School of Public Health)
Certificate, Teaching

8/2006 Duquesne University, Pittsburgh, PA
M.S., Biology

8/2003 University of Pittsburgh, Pittsburgh, PA
B.S., Biology
Certificate, Conceptual Foundations of Medicine

COURSES

8/2014 National Human Genome Research Institute, Bethesda, MD
(Confirmed) Selective Course: Advances in Genomics Research

7/2008 Jackson Laboratory, Bar Harbor, ME
Selective Course: Short Course on Medical and Experimental
Mammalian Genetics

9/2006- Foundation for Advanced Education in the Sciences Graduate School
5/2007 (at the NIH), Bethesda, MD
Course: Introduction to Medical Genetics

7/2001 Ometepe Biological Field Station, San Ramon, Ometepe Island,
Nicaragua
Course: Primate Behavior and Biology

TRAINING

- 9/2013-5/2014 Preparing Future Faculty Teaching Academy, Johns Hopkins University, Baltimore, MD
- 7/2013 Board of Directors Pipeline Leadership Development Project, Associated Black Charities, Baltimore, MD
- 7/2012-Present Academy for Future Science Faculty, Northwestern University School of Medicine, Chicago, IL

HONORS AND AWARDS

- 2014 AACR-Bristol-Myers Squibb Oncology Scholar-in-Training Award
- 2012 Biomedical Scholars Association Outstanding Biomedical Scholar Award
- 2012 Johns Hopkins Martin Luther King, Jr. Award for Community Service
- 2011 Johns Hopkins SOURCE Community Service Award
- 2010-2012 Johns Hopkins Social Innovation Lab Sponsorship
- 2009-2011 John Hopkins Institutions Diversity Leadership Council Appointment (by JHU President)
- 2006-2007 NIH Technical Intramural Research Training Award
- 2005 American Society for Microbiology 1st Place Graduate Student Poster Award
- 2000 National Society of Collegiate Scholars Induction

GRANTS AND FELLOWSHIPS

- 2011 Baltimore Albert Schweitzer Fellowship
- 2004-2006 Duquesne University Graduate Teaching Fellowship
- 2004-2006 Duquesne University Tuition Scholarship
- 2002 Howard Hughes Medical Institute Summer Internship
- 2001 University of Pittsburgh Study Abroad Scholarship
- 1999-2003 University of Pittsburgh Challenge Academic Merit Scholarship

PUBLICATIONS

- 2014 **Mitchell, K.A.**, Noor, M. Easwaran H., Cai Y., O'Hagan H., Baylin S.B. DNMT3B Epigenetically Regulates Gene Expression, Independent of DNA Methylation. Manuscript in preparation.
- 2011 **Mitchell, K.A.** et al. Maryland Comprehensive Cancer Control Plan - Cancer Disparities Chapter Co-Author [Social Determinants of Health Section Author] Baltimore, MD: Department of Health and Mental Hygiene, 16p. Published.

BIOLOGICAL RESEARCH EXPERIENCE

- 6/2008-7/2014 Johns Hopkins University School of Medicine, Baltimore, MD
Ph.D. Student
Area: The role of DNA methyltransferases and epigenetic proteins on transcriptional mechanisms in human cancer
Advisor: Stephen B. Baylin, M.D.
- 8/2006-6/2007 National Institutes of Health-National Human Genome Research Institute, Bethesda, MD
Trainee
Area: Modeling human diseases in zebrafish using an RNA aptamer-based *in vivo* split-protein complementation system
Advisor: Benjamin Feldman, Ph.D.
- 8/2004-8/2006 Duquesne University, Pittsburgh, PA
M.S. Student (Thesis Option)
Area: Quantitative analysis of microbial gene expression during the cold shock response
Advisor: Nancy J. Trun, Ph.D.
- 6/2004-12/2004 University of Pittsburgh Cancer Institute, Pittsburgh, PA
Research Laboratory Volunteer
Area: Chromosome breakage analysis in squamous cell carcinoma of the head and neck
Advisor: Susanne Gollin, Ph.D.
- 7/2001 Ometepe Biological Field Station, San Ramon, Ometepe Island
Field Researcher
Area: Resource utilization of mantled howling monkeys living in a Nicaraguan tropical rain forest
Advisor: Linda Winkler, Ph.D.

PUBLIC HEALTH PRACTICE EXPERIENCE

- 1/2013-7/2014 Johns Hopkins Center to Reduce Cancer Disparities, Baltimore, MD
Trainee
Participated in the Community Advisory Group and Biospecimen Training Network.
Speaker
Supported disparities research projects through community education seminars.
- 8/2011-12/2011 Maryland Department of Health and Mental Hygiene, Baltimore, MD
Office of Minority Health and Health Disparities Intern
Worked to promote health equity amongst various racial and medically underserved groups throughout Maryland.

- 5/2011-9/2013 American Psychological Association Socioeconomic Status Related Cancer Disparities Program, Baltimore, MD
National Translation & Dissemination Network Member
Shared strategies, resources, best practices, and evaluation and reporting tools with cancer-serving organizations and stake holders.
- 5/2011-Present Maryland Cancer Collaborative, Baltimore, MD
Cancer Disparities Committee Member
Proposed utilization and implementation of the Maryland Comprehensive Cancer Control Plan in regard to cancer disparities.
- 5/2010-8/2010 Maryland Comprehensive Cancer Control Plan, Baltimore, MD
Cancer Disparities Chapter Committee Member
Outlined goals, generated ideas, and identified strategies to reduce cancer burden for individuals, healthcare providers, and organizations in Maryland.

EDUCATION EXPERIENCE

University

- 2/2014-4/2014 Johns Hopkins University, Baltimore, MD
Online Teaching Assistant (University Teaching 101)
Assisted with course planning and syllabus design, developing assessments, differentiating teaching strategies, and IT platform management for an asynchronous online course.
- 1/2010-5/2010 Notre Dame University of Maryland, Baltimore, MD
Associate Faculty (Genetics Lab)
Enhanced instructional lessons and enriched activities from prior Genetics Lab curriculum, and designed new content lectures about recent and relevant topics in a private and predominantly female liberal arts college.
- 8/2004-8/2006 Duquesne University, Pittsburgh, PA
Teaching Assistant (Microbiology Lab for Nursing & PA Majors; General Biology Lab I & II)
Responsible for independent weekly lesson planning and teaching, designed and graded assessments, and held weekly office hours in a private liberal arts college.

K-12

- 6/2012-6/2013 Baltimore City Public Schools, Baltimore, MD
Curriculum Designer (Health Sciences, Research and Ethics in Healthcare)
Designed customized research and health curriculum for an urban public high school specializing in health professions.

11/2007-3/2008 Student National Medical Association, Baltimore, MD
Health Professions Recruitment and Exposure Program Coordinator
Planned and implemented a new curriculum for an 8-week high school enrichment program for underserved high school students.

6/1999-4/2001 Jumpstart, Pittsburgh, PA
AmeriCorps Team Leader
Supervised and trained a teaching team of 7 undergraduate AmeriCorps members and 8 disadvantaged preschool children in an urban public school classroom, instructed students using the HighScope teaching approach and curriculum.

Relevant Teaching Coursework

2011-2013 Preparing Future STEM Faculty to Meet the Needs of Culturally and Linguistically Diverse Populations
Emphasis: Learners from diverse backgrounds

Institute for Excellence in Education Summer Teaching Camp
Emphasis: Adult learners in clinical and non-clinical settings

Teaching at the University Level
Emphasis: Undergraduates and graduate students

Introduction to Effective Instruction
Emphasis: K-12 and undergraduates

MENTORSHIP EXPERIENCE

7/2008-Present Junior Research Scholars, Inc., Baltimore, MD
Founder and President/CEO
Launched a science mentorship, college-readiness, and career-readiness program for disadvantaged youth; 18 mentees have collectively participated in 11 scientific competitions, winning a combined 23 ranked spots and special awards.

Relevant Mentorship Coursework

2012 Research Mentor Training
Emphasis: Undergraduate, graduate, lab trainees

Business of Academic Biomedical Research
Emphasis: Undergraduates, graduate students, and lab trainees

Teaching and Mentorship Portfolios Available Upon Request.

ORGANIZATIONAL LEADERSHIP EXPERIENCE

- 11/2011-1/2012 Johns Hopkins Medicine Collaborative Planning Team, Baltimore, MD
Invited Member
Served on a senior executive planning team, comprised of 20+ diverse faculty and staff leaders representing every Johns Hopkins Medicine division, which developed six priorities for the most recent 5-Year Strategic Plan.
- 8/2009-6/2011 Johns Hopkins Diversity Leadership Council, Baltimore, MD
Selected Member
Worked alongside 50+ students, faculty, and staff from all Johns Hopkins divisions to develop institution-wide recommendations that help achieve diversity and inclusion goals. Concurrently served on the Community Partnerships and Recruitment and Retention subcommittees.
- 7/2008-6/2011 Johns Hopkins Biomedical Scholars Association, Baltimore, MD
Community Service Chair
Established the Spring into Science Initiative, an annual community based science outreach program designed to introduce and expose youth in non-traditional target populations to STEM fields.
- President
Implemented active group membership criteria and benefits, attended bi-weekly meetings with the Dean for Student Diversity, established the Women in Science Tea annual event, successfully petitioned for funds and managed an operating budget of \$5,650.
- Programming Vice-President
Established the Diverse Careers in Science Seminar Series, BSA Annual Lecture, Milestone Celebration, and BSA Big Sib Program, expanded official student group status to include JHU Schools of Public Health and Nursing.

SCIENTIFIC POSTER PRESENTATIONS

- 8/2014
(Confirmed) National Human Genome Research Institute Advances in Genomics Research Summer Program
Bethesda, MD
"DNMT3B (a *de novo* DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity"
- 2/2014 Burroughs Wellcome Fund Maryland Genetics, Epidemiology and Medicine Genetics Research Day
Baltimore, MD
"DNMT3B (a *de novo* DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity"

- 11/2006 National Human Genome Research Institute Annual Scientific Retreat
Gettysburg, PA
"Visualizing RNA expression in early zebrafish embryos: a pilot study"
- 11/2005 American Society for Microbiology Allegheny Branch Fall Meeting
Clearfield, PA
"An Investigation of the mRNA Abundance and Expression Patterns for the Nine *csp* Genes in *E. coli* K-12"
- 10/2005 University of Pittsburgh Science 2005: The New Research Ecology
Pittsburgh, PA
"An Investigation of the mRNA Abundance and Expression Patterns for the Nine *csp* Genes in *E. coli* K-12"
- 4/2002 American Association for Physical Anthropology 71st Annual Meeting
Buffalo, NY
"Adaptive strategies and resource utilization of the mantled howling monkey (*Alouatta palliata*) in a small forest fragment in Nicaragua"

SCIENTIFIC ORAL PRESENTATIONS

- 6/2014 2014 Geographical Management of Cancer Health Disparities Program
Conference
Bethesda, MD
a) "Trainees Becoming Independent Researchers: Experiences, Challenges, and Opportunities"
b) "DNMT3B (a *de novo* DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity"
- 4/2014 American Association for Cancer Research 105th Annual Meeting
San Diego, California
"DNMT3B (a *de novo* DNA methyltransferase) epigenetically regulates gene expression, independent of its DNA methyltransferase activity"
- 10/2011 Johns Hopkins Institute of Genetic Medicine, Departmental Seminar
Baltimore, MD
"Determining the relationship between the cancer-associated metabolite 2-hydroxyglutarate (2HG), TET proteins, and the CIMP phenotype in colorectal and breast cancer"

STEM EDUCATION PRESENTATIONS

- 10/2014 International Black Doctoral Network Association Annual Conference
(Confirmed) Philadelphia, PA
Selected Speaker
"STEMsational Mentoring: A Transformative Program Model For Increasing STEM Interest in College-Bound Urban Youth"

- 6/2014 Smithsonian National Museum of Natural History Genome Zone Program
Washington, DC
Featured Scientist
"Genome Geeks: Epigenetics"
- 1/2014 Johns Hopkins University Science of Learning & Gateway Sciences Symposia
Baltimore, MD
Selected Poster
"From The Block To The Bench: Transforming Disadvantaged Baltimore City High School Students Into Science Scholars"
- 12/2013 Wheaton High School Bioscience Program
Silver Spring, MD
Invited Speaker
"Cancer and genetics and epigenetics! Oh my!"
- 10/2013 International Black Doctoral Network Association Annual Conference
Philadelphia, PA
Selected Speaker
"From The Block To The Bench: Transforming Disadvantaged Baltimore City High School Students Into Science Scholars"
- 5/2013 Action in Maturity Senior Center
Baltimore, MD
Invited Speaker
"Biospecimens, Genetics, and Cancer"
- 6/2012 Johns Hopkins Berman Institute of Bioethics Outreach Program
Baltimore, MD
Invited Speaker
"How Henrietta Lacks Helped Me Get A Ph.D."
- 10/2010 Johns Hopkins University Executive Alumni Council
Baltimore, MD
Invited Speaker
"Spring into Science at the House of Ruth"
- 9/2003 University of Pittsburgh Office of Experiential Learning
Pittsburgh, PA
Invited Speaker
"Monkeys, Fig Newtons, and Me: Surviving a Nicaraguan Rain Forest"

PROFESSIONAL SERVICE**City**

5/2013- Johns Hopkins School of Public Health "B'More Healthy: Communities
5/2014 for Kids" Study
Recreation Center/Peer-Mentor-Level Working Group Member

9/2012- Morgan State University Math-Science-Engineering Fair
Present Steering Committee Member

State

4/2013- Maryland Governor's Office on Service and Volunteerism
5/2013 AmeriCorps Grant Reviewer

National

7/2012- U.S. Department of Health and Human Services-Health Resources and
Present Services Administration
Federal Field Grant Reviewer

4/2007 National Institutes of Health-National Human Genome Research
Institute
DNA Day Ambassador

2004- American Society of Human Genetics, Genetics Education Outreach
Present Network Member
State Science Standards and Content Reviewer

PROFESSIONAL MEMBERSHIPS

2010- American Association for Cancer Research
Present

2010- Association for Women in Science
Present